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CANCER RESEARCH

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Localization and Integration of Cellular Function*

GEORGE H. HOGEBOOM, WALTER C. SCHNEIDER, AND MARY JO STRIEBICH

(National Cancer Institute, National Institutes of Health, Public Health Service, Bethesda, Md.)

For many years, the cytologist has had reason to believe that the complex structural organization of the cell, which was demonstrated so beautifully by the previous speakers, is a reflection of an equally complex chemical organization. It can be argued, in fact, that cellular architecture evolved in such a way as to permit an orderly sequence of metabolic processes and thus to avoid interference and competition among the many biochemical reactions that occur simultaneously. It follows, therefore, that specific cellular structures should have specific chemical properties. That these ideas are not simply figments of the imagination is becoming increasingly apparent from data obtained in a relatively new and still somewhat controversial area of research, now generally referred to under the term, cytochemistry.

Cytochemistry is at present strictly in a methodological stage. Although several general methods of approach to cytochemical problems have been devised, it is an unfortunate fact that only one is capable, even in theory, of keeping pace with the rapid advances in biochemistry. We are referring to the actual isolation of cell structures and the direct determination of their biochemical properties. The situation is unfortunate, because this technic is by no means perfect and cannot be checked readily by other methods of attack. In this discussion, we propose, therefore, to take the only reasonable alternative—i.e., to examine critically the data that are available and, with the defects of our technic in mind, to attempt to arrive at conclusions that are based on sound experimentation.

* Presented April 10, 1953, to the American Association for Cancer Research as part of a symposium entitled "The Morphology and Activities of Living Cells and Cellular Components."

The main inspiration for the use of the cell fractionation technic as a cytochemical tool has been the pioneer work of Claude (19–22), who devised systematic procedures for the isolation of cytoplasmic elements by means of the differential centrifugation of broken cell suspensions. Although Bensley and Hoerr (7) had previously reported the isolation of liver mitochondria, the details of these experiments were not published until a number of years later. Since 1948, the methods of Claude have been considerably improved (52, 53, 95, 97, 99), and extensive data relating to the biochemical properties of cell structures have accumulated.

In general, the isolation of cell structures is accomplished first by the preparation of tissue "homogenates" under conditions leading to minimal alteration of cell components and subsequently by the fractionation of the particulate structures on the basis of their differential rate of sedimentation in the centrifuge. In the case of mammalian liver, the application of increasing centrifugal forces yields four fractions: cell nuclei, mitochondria, microsomes or submicroscopic particles, and a supernatant consisting mainly of compounds in solution.

THE CELL NUCLEUS

The role of the nucleus in cellular metabolism is probably the most controversial subject with which we have to deal. The story behind the present, somewhat diverse concepts of nuclear biochemistry is, however, an interesting one, since it illustrates practically every difficulty encountered in cell fractionation technics, with respect both to the methods employed and to the interpretation of the data. With your permission we should therefore like to go into this story in some detail.

Obviously, it is much simpler to define than to devise an adequate method for the isolation of cell nuclei. It should provide unaltered nuclei in homogeneous preparations and in good yield. In using the term "unaltered," we are referring to the absence of both morphological and chemical changes. Thus, it is difficult to believe that a cytologically altered nucleus could possibly be chemically intact. On the other hand, the converse proposition, that a cell nucleus appearing to be cytologically intact is therefore chemically unaltered, does not follow. In this respect, we must constantly bear in mind the fact that the conditions under which cell fractions are isolated are far from physiological and that a normal microscopic appearance of isolated structures does not eliminate the possibility of such artifacts as denaturation, extraction and redistribution, or adsorption of proteins. It appears to us that the answer to the difficult problem of detecting chemical alterations will depend on the objective, critical evaluation of extensive data, on a continuous search for better methods, and on a diligent alertness for the occurrence of artifacts.[1]

The necessity for striving for homogeneity is obvious. We must go even farther than this, however, for the term "homogeneity" can only be used in a relative sense, and quantitative methods of analysis must therefore be devised for determining the degree of homogeneity attained. This is an axiom in the field of chemistry. In cytochemistry and in the absence of a better method, microscopic examination has usually been employed as the chief means of estimating the purity of preparations of isolated cell structures. Unfortunately, microscopic examination is not in itself an objective, quantitative method and, in our opinion, can only be used as a means of ruling out gross contamination. On this basis we have taken the stand that the assignment of a biochemical property to the cell nucleus can be safely made only when that property is present in isolated nuclei in a relatively high concentration—for example, in a concentration exceeding that in the whole tissue (42, 99). This principle, which also applies to any other cell structure, is obviously arbitrary and will certainly have to be abandoned when improved methods are available.

The question of yield is also of importance. To isolate 5 per cent of the nuclei of a tissue and assume that analyses of that preparation are representative of all the nuclei is not a sound procedure. Thymus, a tissue often used as a source of cell nuclei, is made up of cells containing nuclei of widely different appearances and perhaps therefore of different chemical composition. It is entirely possible that an isolation procedure leading to a low yield may at the same time result in fractionation of the different types of nuclei.

Another important point relating to the question of yield arises from the fact that a high yield makes it possible to determine the proportion of the enzyme activity of the whole tissue recovered in each cell fraction. Recovery values can be very enlightening. Many enzymes that are said to reside in the liver cell nucleus have been found in isolated preparations in a concentration of 40 to 100 per cent of that in the whole tissue (26–28). When it is realized that the nucleus of the liver cell accounts for about 10 per cent of the total mass of the cell, it is apparent that these concentration values of 40–100 per cent would have represented enzyme recoveries of only 4–10 per cent *if* the yield of nuclei had been complete, and in these experiments the yield of nuclei was far from complete. It is our opinion that cell fractionation procedures are fraught with too many uncertainties to permit definitive conclusions in the face of enzyme recoveries of this order.

Attention to yield and recovery also makes it possible to draw up a balance sheet, a point that we have for some reason been forced to defend on numerous occasions (42, 99; cf. 32, 111). It is not enough to determine the enzyme activity of a single fraction with the object of interpreting that analysis in terms of the whole tissue. The original whole tissue and all fractions must be analyzed. If the sum of the activity of the fractions equals within reasonable limits the activity of the whole tissue, then the method of enzyme assay is likely to be adequate, at least for cytochemical purposes. If not, something is wrong with the enzyme determination.[2]

[1] An excellent example is found in the work of Novikoff (77, 78), who has shown that nuclear material has a strong affinity for calcium phosphate. This finding has demonstrated that the previously accepted and widely used histochemical method for the localization of alkaline phosphatase is capable of producing an artifact and is therefore useless as a means of investigating the intracellular locale of the enzyme.

[2] The necessity for drawing up balance sheets becomes obvious when one considers the inherent difficulties involved in enzyme assays, especially in dealing with complicated mixtures of unknown biochemical composition. Enzyme assays are based on determinations of reaction rates. Reaction rates can be markedly affected by a number of factors, including inhibitors, activators, interference or competition as a result of side reactions, relatively slight changes in the pH or ionic strength of reaction mixtures, and permeability barriers set up by membranes. Unfortunately, one of the most common sources of misleading data, particularly in certain investigations of complex enzyme systems, has arisen from the probability that the enzyme supposedly under study was not the rate-limiting component of the reaction. In many if not most instances, any one of these difficulties can be detected by the analysis of all cell fractions and the use of balance sheets.

A number of methods have been devised for the isolation of cell nuclei. Most have given little attention to yield but, with some justification, have been aimed at obtaining homogeneous preparations. In some investigations, analyses have been confined solely to the preparations of isolated nuclei; in only a few have the data been sufficiently complete to permit a comparison of analyses of the nuclei with those of the whole tissue and cytoplasmic fractions. In general, concrete data relating to the purity of the preparations have been lacking.

In an attempt to obtain reasonably homogeneous preparations of liver cell nuclei in good yield, a survey was made in our laboratory of several of the isolation technics available. Of the methods considered, all led to one or more of the following difficulties: low yields, impure preparations, denaturation or extraction of proteins, and damaged or otherwise alterated nuclei.[3] That of R. M. Schneider and Petermann (93) seemed, however, to offer the best possibilities. These investigators found that the addition of a small amount of $CaCl_2$ to homogenates of lymphoid tissues prepared in sucrose solutions prevented the aggregation of nuclei, apparently made them resistant to mechanical injury, and did not adversely affect their morphology.

In applying this observation to the isolation of liver cell nuclei, it was necessary to eliminate, insofar as possible, three major sources of contamination; connective tissue, intact liver cells, and free mitochondria. It was also necessary to devise quantitative methods for assessing the homogeneity of the preparations. Toward this end technics were developed for determining by direct count under the phase microscope the number of liver cells, free nuclei, and free mitochondria in homogenates and cell fractions (53, 104).

A relatively simple procedure (53) making it possible to obtain nuclei in high yields and in reasonably homogeneous preparations is shown in Chart 1. Perfused livers were homogenized with a Potter-Elvehjem type homogenizer in 10 volumes of an isotonic sucrose-$CaCl_2$ solution. The homogenate was filtered to remove connective tissue and layered over 2 volumes of a slightly stronger sucrose solution containing a lower $CaCl_2$ content, thus forming a "two phase" system. When the mixture was centrifuged for 10 minutes at $600 \times g$ in a horizontal centrifuge, the nuclei and residual intact liver cells were completely sedimented, whereas almost all the mitochondria remained above the interface. The entire supernatant was withdrawn, and the pellet was rehomogenized in order to disrupt the remaining unbroken cells. The layering procedure and centrifugation were then repeated. Table 1 shows typical analytical data obtained after a total of four such centrifugations. The yield of nuclei, as shown by DNA phosphorus analyses, was 79 per cent, and the preparations of nuclei contained only 0.33 per cent of the free mitochondria of the homogenate and 0.54 per cent of the intact cells of the original whole tissue. Although the latter figures would seem to be insignificantly low, it should be pointed out that about 6 per cent of the nitrogen of these preparations was non-nuclear, mainly as a result of the presence of the intact liver cells. It is obvious, therefore, that the extent of contamination must be small and must be known before too much reliance can be placed on chemical analyses of such preparations.

Photomicrographs of the nuclei isolated by this technic have been published elsewhere (53). In general, the individual nuclei appear to be in reasonably good shape. The nuclear membranes and nucleoli are unusually prominent, but no other clearly defined structure is visible. The nuclei are not shrunken, and the coarse precipitation patterns generally seen in nuclei isolated by several other accepted technics are notably absent. Some of the nuclei are damaged, however, as shown by a distorted shape or a tear in the nuclear membrane. A structure having the appearance of a collapsed cell membrane is also present in the preparations (53). At present we can give no quantitative estimate of the analytical error introduced by this contaminating element but can only say, rather lamely, that the cell membranes are greatly outnumbered by the nuclei.

Despite certain defects, this procedure for the isolation of liver cell nuclei and for the quantitative estimation of the major sources of contamination of the preparations has permitted a reinvestigation of enzymes that have previously been considered important constituents of the cell nucleus. An example is cytochrome oxidase, which is present in much higher concentrations in the mito-

[3] The use of aqueous citric acid at pH 4 (26) was obviously unsuitable for enzyme studies because of the acidity of the medium. Citric acid at pH 6 (26), although better in this respect, left something to be desired with respect to yield and nuclear morphology and, according to Dounce (28) leads to exchange of soluble proteins with the cytoplasm. Poor yields were obtained by the method of Wilbur and Anderson (119), who used sucrose solutions containing a phosphate buffer at neutral pH. The fragility of nuclei and their tendency to aggregate in the sucrose solutions suitable for the fractionation of cytoplasmic elements made it impossible to obtain a nuclear fraction of satisfactory purity with these media (99). Later modifications of the sucrose technic, introduced recently by Lang and Siebert (63) and by Chauveau (17), were not published until after the present survey was completed.

chondrion, which in turn is one of the most likely contaminants of isolated nuclei. As shown previously, when nuclei and mitochondria are adequately separated, the cytochrome oxidase activity of the isolated nuclei becomes vanishingly low, and the small amount of activity remaining is closely correlated with the number of free mitochondria present (53). A similar situation was found to exist with respect to the enzyme, uricase (53). Obviously, these experiments are, in a sense, negative and only serve to cast doubt on previous conclusions concerning the role of the nucleus in cellular metabolism. Before mentioning a few positive results, however, we should like to discuss briefly a somewhat different approach to the study of the chemistry of the cell nucleus.

A rather ominous source of possible serious difficulty in the isolation of nuclei arises from considerations of the permeability of the nuclear membrane. Thus, it is conceivable that the membrane is sufficiently permeable to permit the extraction of water-soluble material by aqueous media. To overcome this objection, Behrens in 1932 (5) devised a laborious and time-consuming procedure for the isolation of nuclei, the steps in the isolation consisting of freezing, desiccation, and grinding of the tissue and the subsequent centrifugal separation of nuclei from a mixture of organic solvents of low polarity.[4] The analyses of nuclei

[4] This procedure is obviously unsuitable for use as a general technic in enzyme studies. Nevertheless, some enzymes apparently do survive it, at least to some extent, and it has recently been modified slightly by Allfrey *et al.* (1) and used recently by the same group of investigators (111) in the study of a number of enzyme reactions. Although, as stated by Allfrey *et al.*, the extraction of water-soluble proteins from the nuclei is undoubtedly eliminated by the Behrens technic, it should be pointed out conversely that the evidence presented by these investigators (1) for the extraction of proteins by *aqueous* media is not convincing.

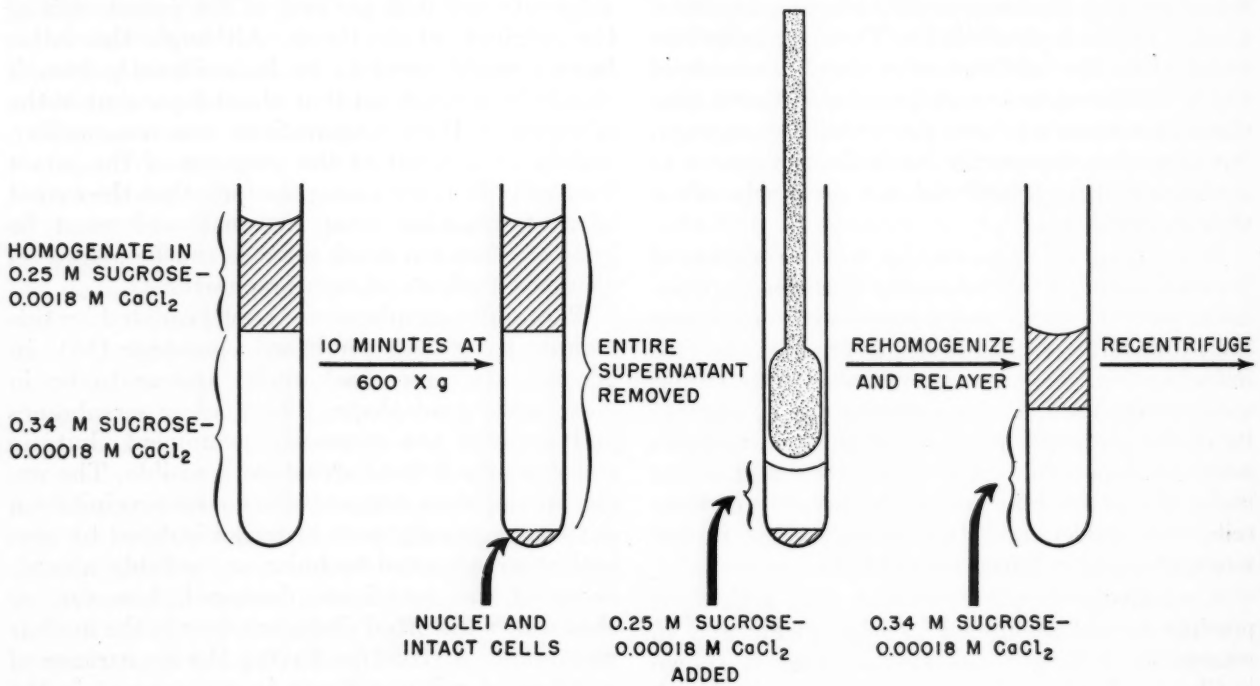

CHART 1.—The isolation of cell nuclei in an aqueous medium

TABLE 1

ISOLATION OF CELL NUCLEI IN SUCROSE-$CaCl_2$ SOLUTIONS

Preparation	Total nitrogen	Desoxypentosenucleic acid phosphorus		Pentosenucleic acid phosphorus		Intact liver cells*	Free mitochondria†
	Per cent of original	Per cent of original	μg/mg of nitrogen	Per cent of original	μg/mg of nitrogen	Per cent of original	Per cent of original
Homogenate	100	100	12	100	22	2.5	100
Filtered homogenate	95	87	11	87	20	1.6	98
Isolated nuclei	9.2	79	94	10	23	0.54	0.33
Supernatant	85						

* There are approximately 28×10^7 cells in 1 gm. of liver (53).

† There are approximately 11×10^{11} mitochondria in 1 gm. of liver (53).

isolated by means of the Behrens technic are generally not in agreement with those of nuclei isolate in aqueous media and the differences, as we shall demonstrate, are not easily reconciled. Although the Behrens method possesses the advantage just mentioned, several possible sources of artifacts arising from its use need further investigation.

Table 2 lists those enzymes that have been found in isolated liver cell nuclei in a concentration equal to or exceeding that in the whole tissue. There is, of course, an even longer list of enzymes which were present in lower concentration (26, 111), but, as in the case of cytochrome oxidase and uricase, the latter findings are at present of doubtful cytochemical significance and would only add confusion to this discussion. As a matter of fact, even the data shown in Table 2 are little short of confusing. Thus, if an enzyme were exclusively localized in the nucleus, its concentration in a pure preparation of nuclei would be approximately 8. It is evident, therefore, that, with one exception, well defined localization of enzymes within the cell nucleus is not indicated by these results. In several instances, the results obtained with the Behrens technic are in sharp disagreement with the data obtained with aqueous media, an outstanding example arising from studies of the enzymatic dephosphorylation of ATP. The assay of this reaction is complicated by the fact that there are at least two enzymes involved, the more powerful of which is activated by magnesium ions and concentrated in mitochondria (60, 79). The other is calcium-activated and, from work carried out in several laboratories (79, 102), appears to be localized in the nucleus. Yet the data obtained with the Behrens technic (Table 2) indicate that the reaction is entirely cytoplasmic in origin. Since the ATPases fall into the class of "insoluble" or particulate enzymes, however, it seems unlikely that they would be redistributed readily among cell components after disruption of the cell, and the question arises as to whether the Behrens procedure might have resulted in selective destruction of a nuclear ATPase. Novikoff's finding (77) that ATPase is destroyed by acetone lends support to this suggestion.

In general, several possible conclusions can be derived from the data on nuclear enzymes. It is possible that many of the enzymes studied are exclusively nuclear in origin but were partially lost during the fractionation procedures or, as Dounce has pointed out (27), that sharp localization of enzymes in the nucleus simply does not occur as it

TABLE 2

BIOCHEMICAL PROPERTIES OF LIVER CELL NUCLEI

Enzyme system or compound	Concentration (whole tissue = 1)	Recovery (whole tissue = 100)	Method of isolation	Reference	Remarks
D-Amino acid oxidase	1.0	?	Citric acid	(26, 27)	
Arginase	1.1	?	Citric acid	(26, 27)	
	2.5	36	Water	(92)	
	0.6	?	Behrens	(111)	
Adenosinetriphosphatase	1.7	31	Sucrose	(102)	Activated by Ca^{++} (79).
	0.1	?	Behrens	(111)	Inactivated by organic solvents? (77).
Adenosine-5-phosphatase	2.9	40	Sucrose	(80)	
	0.2	?	Behrens	(111)	
Alkaline phosphatase	1.9	?	Citric acid	(26, 27)	
	0.1	?	Behrens	(111)	
	0.4	16	Sucrose	(81)	
Adenosine deaminase	1.8	?	Behrens	(111)	90 per cent of activity recovered in soluble fraction (100).
Nucleoside phosphorylase	1.0	?	Behrens	(111)	93 per cent of activity recovered in soluble fraction (100).
	Absent	Absent	Sucrose-phosphate	(111)	
Uricase	1.0	?	Citric acid	(26, 27)	
	0.1	1	Sucrose-$CaCl_2$	(53)	
DPN nucleosidase	?	34(?)	Sucrose	(115)	Over-all recovery = 130–140 per cent.
Synthesis of diphosphopyridine nucleotide (DPN)	5.5	92	Sucrose	(50)	Enzyme released into solution on disruption of nuclei (Cf. 49).
	6.0	69	Sucrose-$CaCl_2$	(50)	
Desoxypentosenucleic acid	7.3–8.1	70–90	Sucrose-$CaCl_2$	(53)	
Pentosenucleic acid	1.0	10	Sucrose-$CaCl_2$	(53)	

does in the cytoplasmic structures. A third possibility must also be borne in mind, namely, that most of the enzymes that are truly nuclear in origin remain to be discovered.

The enzyme catalyzing the synthesis of diphosphopyridine nucleotide or DPN from adenosinetriphosphate and nicotinamide mononucleotide appears to be an outstanding exception to the general run of data obtained in studies of isolated nuclei. Here, both the concentration and recovery values are sufficiently high to indicate exclusive localization of the enzyme in the cell nucleus (50). Now this situation is rather interesting from several angles. The enzyme, which was discovered and partially purified by Kornberg (61), is soluble in water and is released into solution when the nuclei are disrupted (50). Yet it is not extracted from nuclei by aqueous media during the isolation procedure, and it is therefore not necessary to resort to the Behrens method to demonstrate its localization. Since DPN is a coenzyme for many reactions that apparently occur in the cytoplasm, the synthesis of the compound by the nucleus would appear to be an example of a biochemical relationship between nucleus and cytoplasm and thus of a means of nuclear control over cytoplasmic function. Microscopic (15) and *in vivo* studies with labeled precursors (71, 88) have indicated that ribonucleic acid may also be synthesized in the nucleus. When it is remembered that DPN is a ribose dinucleotide, a possible analogy between these two findings becomes apparent.

As Stern and Mirsky (112) have implied, whether the chromosomes function in an energy-yielding environment is a question of great importance. The apparent absence from the nuclei of cytochrome oxidase and other enzymes carrying out oxidative reactions would seem to eliminate this possibility. If the enzymes taking part in glycolysis are present in animal nuclei, they must be extracted during the isolation procedures employed. The nuclei of wheat germ apparently do contain glycolytic enzymes (112), but this finding may represent a special case, since wheat germ nuclei account for a very high proportion of the total mass and ribonucleic acid of the whole tissue (112). The dephosphorylation of ATP is, of course, an energy-yielding reaction, and it has been proposed by Lang and Siebert (64) that the energy requirements of the nucleus could be provided by the ATPase present. In this respect and also in regard to the synthesis of DPN, it is of interest to note that mitochondria are able to synthesize ATP at a very rapid rate. It is possible, therefore, that ATP is synthesized in the cytoplasm and is transferred to the nucleus to take part in further reactions at that site.

The Mitochondrial Fraction

In studying the properties of isolated mitochondria, we find ourselves, in some respects, on firmer ground than when dealing with isolated nuclei. Thus, the mitochondrion possesses a surface membrane that, as we shall demonstrate, is capable of retaining not only soluble proteins but also compounds of low molecular weight. The extraction of water-soluble material that is said to occur during the isolation of nuclei in aqueous media does not, therefore, appear to be a serious menace in the isolation of mitochondria. There are, however, other problems. Mitochondria are small and not easily identified microscopically, being recognized chiefly by their affinity in the unfixed state for Janus Green B, a transient property that is demonstrated with difficulty, and by their staining reactions with certain other dyes after special types of fixation (52). An estimation of the homogeneity of mitochondrial preparations is therefore not readily made and is dependent mainly on microscopic examination.[5]

In general, difficulties similar to those encountered in determining the homogeneity of preparations of isolated nuclei have led us to question the significance of a relatively low concentration of an enzyme in the mitochondrial fraction (99). Both cytological and biochemical studies have indicated, however, that the use of certain nonionic aqueous media in the fractionation of liver makes it possible to obtain mitochondria in preparations that are not grossly contaminated by extraneous material (52, 99). Under these conditions, the yields are greater than 70 per cent. Thus, although further studies of the homogeneity of the mitochondrial fraction are unquestionably in order,[6] there is reason to believe at present that a relatively high concentration and recovery of a biochemical property in the fraction is of cytochemical significance.

Tables 3–5 list the enzymes and related compounds that appear, on the basis of investigations carried out in a number of laboratories, to be localized in liver mitochondria. Since time does not

[5] Studies by Zollinger (122, 123) and by Harman (33) have, however, recently provided another means of identification of mitochondria. When exposed to media of low tonicity, the particles become swollen and, in the phase microscope, show a crescent-shaped mass of dense material at one pole. This characteristic is made use of in the method of counting mitochondria mentioned earlier.

[6] Recent investigations of the biochemical homogeneity of isolated mitochondria have been reported by Novikoff *et al.* (81) and by Schneider (96).

TABLE 3

BIOCHEMICAL PROPERTIES OF LIVER MITOCHONDRIA

Enzyme system or compound	Concentration (whole tissue = 1)	Recovery (whole tissue = 100)	Reference	Remarks
Cytochrome oxidase	3.1	79	(98)	* (cf. 43, 53, 94)
Cytochrome c	2.3	51	(97)	* Most of remainder in soluble fraction (cf. 6).
DPN-cytochrome c reductase	1.2	28	(44)	* Most of remainder recovered in microsomes (cf. 41).
TPN-cytochrome c reductase	2.3	49	(46)	Also concentrated in microsomes.
Succinic dehydrogenase	2.8, 2.4	72, 70	(52, 98)	* Partially inactivated by damage to mitochondria (45), (cf. 43, 94).
α-ketoglutaric oxidase	1.5	20	(106)	Mitochondrial activity enhanced by the addition of other fractions (cf. 56).
Oxalacetic oxidase	1.8	45	(103)	Mitochondrial activity enhanced by the addition of other fractions.
Octanoic oxidase	2.3	80	(95)	Inactivated by damage to mitochondria (45), (cf. 56).
Glutamic dehydrogenase	3.3	83	(51)	† Activity enhanced by damage to mitochondrial membranes.
β-hydroxybutyric dehydrogenase	?	?	(67)	Data incomplete.
Synthesis of citric acid	?	?	(54)	Data incomplete. See evidence from *in vivo* experiments described in text.
Synthesis of ATP (oxidative phosphorylation)	6.7(?)	94(?)	(106)	Assay method subject to interference by ATPase (cf. 23, 59, 67, 75, 87).
Adenylate kinase	?	72	(59)	†
Adenosinetriphosphatase	2.2, 2.4, 2.5	50, 64, 50	(102, 79, 81)	* Activity enhanced by damage to mitochondrial membranes (59). Activated by Mg^{++}

* After disruption of mitochondria, enzyme remains attached to sedimentable particles.
† After disruption of mitochondria, enzyme goes into solution.

TABLE 4

BIOCHEMICAL PROPERTIES OF LIVER MITOCHONDRIA

Enzyme system	Concentration (whole tissue = 1)	Recovery (whole tissue = 100)	Reference	Remarks
Choline oxidase	3.1	78	(57)	(cf. 120)
Betaine aldehyde oxidase	?	50	(121)	
D-Amino acid oxidase	?	?	(20)	Data incomplete.
Tyramine oxidase	2.2, ?	57, 67	(24, 37)	
α-glycerophosphate dehydrogenase	?	60(?)	(25)	Method of enzyme assay probably inadequate.
Catalase	?	45	(70)	Remainder of activity in soluble fraction.
Uricase	2.5, 3.0	74, 65	(91, 100)	* Particles containing uricase may not be mitochondria (81), (cf. 96).
Rhodanese	?, 4.2	62, 67	(70, 108)	
Cathepsin	2.6	46	(72)	
Glutaminase I	3.7	78	(105)	
Acid phosphatase	1.3, ?	42, 61	(83, 9)	† Activity enhanced by damage to mitochondrial membranes (8, 29).
Adenosine-5-phosphatase	?, 0.5	40–45, 15–20	(80–81)	Also concentrated in nuclear fraction.
Ribonuclease	2.2	58	(101)	† Activity enhanced by damage to mitochondrial membrane.
Desoxyribonuclease	2.7	73	(101)	† Activity enhanced by damage to mitochondrial membrane.
Glutamic-phenylpyruvic transaminase	?	100(?)	(40)	Data incomplete.
Glutamic-oxalacetic transaminase	2.3	22	(76)	
Synthesis of:				
p-aminohippuric acid	2.8	90	(58)	
hippuric acid	?	?	(69)	Data incomplete.
citrulline	?	?	(75)	Data incomplete.

* After disruption of mitochondria, enzyme remains attached to sedimentable particles.
† After disruption of mitochondria, enzyme goes into solution.

permit a detailed discussion of all these data, we shall limit our comments to the findings that seem to be most interesting at the moment.

The mitochondria account for about 25 per cent of the total mass or nitrogen of rat or mouse liver (99). Their most striking biochemical property (Table 3) is their content of enzyme systems related to the respiratory activity of the cell. As indicated earlier, the utilization of the molecular oxygen in the oxidation of reduced cytochrome c, that is, the terminal respiratory enzyme system, or cytochrome oxidase, appears to be localized exclusively in mitochondria. The significance of this finding becomes evident when it is realized that the ultimate source of energy for all the activities of the cell is mainly provided by this reaction. It is not surprising, of course, that systems closely related to cytochrome oxidase should also be found in the mitochondrial fraction. Thus the particles contain a high concentration of cytochrome c and are capable of transferring electrons between this compound and the pyridine nucleotides, DPN and TPN. Mitochondria play an important role in the Krebs cycle series of reactions, as indicated by the presence in the fraction of succinic dehydrogenase and by the oxidation of α-ketoglutarate, oxalacetate, and octanoate. Although the data are less complete in studies of the synthesis of citrate by the condensing enzyme of Stern and Ochoa (113), this reaction would also appear to be a function of mitochondria. Glutamic and β-hydroxybutyric acid dehydrogenases, enzymes requiring a pyridine nucleotide as a coenzyme and related to the Krebs cycle, are also present.

TABLE 5

BIOCHEMICAL PROPERTIES OF LIVER MITOCHONDRIA

Compound	Concentration (whole tissue = 1)	Recovery (whole tissue = 100)	Reference
Vitamin B_6	1.3	33	(90)
Vitamin B_{12}	2.2	56	(117)
Riboflavin (total)	1.8	53	(89)
Flavin adenine dinucleotide	2.3	65	*
Folinic acid	1.8	38	(117)
Pantothenic acid	?	43	(39)
Coenzyme A	?	52	(39)
Citric acid	4.4	67	†
Phospholipid	1.5, 1.8	27, ?	(94, 116)

* W. C. Schneider and G. H. Hogeboom, unpublished.

† W. C. Schneider, M. J. Striebich, and G. H. Hogeboom, unpublished; damage to mitochondrial membrane releases citric acid into solution.

A reaction that has an important bearing on the role played by the mitochondrion in the integrated activities of the cell is the synthesis of adenosinetriphosphate through oxidative phosphorylation. In the presence of an oxidizable substrate, such as α-ketoglurate, glutamate, or succinate, adenosinediphosphate is converted to ATP at an extremely rapid rate by isolated mitochondria (59). The presence of adenylate kinase, which establishes an equilibrium among the three adenylic compounds, makes it possible for mitochondria also to convert adenosine-5-phosphate to ATP. The complexity of the oxidative phosphorylation reaction and the fact that it is subject to interference by side reactions makes difficult a definitive study of its intracellular distribution. The data of Siekevitz (106) indicate, however, that it may be an exclusive function of mitochondria. A magnesium-activated adenosinetriphosphatase, that removes only the terminal phosphate from ATP, is also concentrated in this fraction but, as shown by Kielley and Kielley (59), is inactive when the mitochondria are freshly isolated in isotonic sucrose solution. The latter investigators (60) have also demonstrated that when the mitochondria are disrupted in a Waring Blendor, the ATPase is rendered highly active and remains attached to fragments sedimentable only at high centrifugal forces, whereas adenylate kinase passes into solution, and the oxidative phosphorylation reaction disappears.[7] The means by which the mitochondrion is able to control these three reactions is an interesting problem and may depend on the spatial relationship of the enzyme systems within the particle.

Because of its high energy phosphate bonds, ATP occupies a central position in cellular metabolism, taking part in many reactions that are widely distributed among the various cell structures. ATP has, of course, received considerable attention as a possible source of energy for synthetic reactions. Our present knowledge of the mechanism of biosynthesis of this compound (23, 67, 87) has provided a strong foundation for Claude's suggestion (22) that the mitochondrion is the power plant of the cell.

It is our opinion, however, that a number of conclusions relating to the enzyme content of mitochondria are not backed by adequate data. The result is that the mitochondrion has become so popular that the remainder of the cell would almost seem to be inert. It would be teleologically gratifying, for example, if all the enzymes of the Krebs cycle were localized in this cell structure. In spite of statements to this effect (32), the data indicate that some of these enzymes, isocitric dehydrogenase (46) and aconitase[8] being notable

[7] The finding that the ATPase activity is strongly inhibited by adenosinediphosphate and that mitochondria contain considerable quantities of the latter compound (60) may account, at least in part, for the fact that the intact particles show no ATPase activity.

[8] A. L. Lehninger, personal communication.

examples, are localized in other cell fractions and that the Krebs cycle is an integrated function of several cell structures. Additional evidence for the latter view has been provided by the finding that the oxidation of oxalacetate and α-ketoglutarate by mitochondria is stimulated by the addition of other cell fractions which by themselves are inactive. Much of the confusion concerning the properties of mitochondria has arisen from determinations of oxygen uptake when either isolated mitochondria or heterogeneous particulate preparations such as cyclophorase (32) are incubated in the presence of various substrates. The occurrence of oxygen uptake (and it usually does occur when a sufficiently high concentration of the particulate preparation is used) is often taken as proof of the mitochondrial localization of all the enzymes having to do with the oxidation of each substrate.[9] It is doubtful that such studies, made on single preparations without reference to the whole tissue or to other cell fractions, shed much light on the intracellular locale of the enzymes involved. A sounder approach, and, unfortunately, a much more difficult one, is the *direct* determination of the activity of an individual enzyme and a study of its distribution among *all* cell fractions. Under these conditions, the rate-limiting component of the reaction is known to be the enzyme in question, and the possible occurrence of such artifacts as adsorption can be directly studied.

As shown in Table 4, other enzymes apparently associated with mitochondria are a number of additional oxidases and a miscellaneous group, including phosphatases, possibly transaminases, and several synthetic systems for which complete data are available only in the case of *p*-aminohippuric acid synthesis. It is rather surprising that ribonuclease and desoxyribonuclease should turn up in the mitochondrial fraction, since the native substrates for both of these enzymes are present in other cell structures.[10] Finally, as indicated in Table 5, a number of vitamins and coenzymes are concentrated in the fraction, as well as citric acid and phospholipid.

Before discussing other cell fractions, we should like to mention several additional properties of liver mitochondria that appear to be of basic interest. As pointed out earlier, the particles possess a surface membrane (99, cf. 32). That this membrane is a physiological as well as a morphological entity is indicated by a number of investigations. When the membrane is disrupted, over 50 per cent of the total nitrogen of isolated mitochondria is released into solution (45, 47). Most of the soluble nitrogen is in the form of proteins that have been separated and characterized in the analytical centrifuge. The most prominent component of the mixture has a sedimentation rate of 6.3 Svedberg units (47) and, as indicated by unpublished experiments carried out in our laboratory, apparently is a type of globulin. Of some interest is the finding that this protein could not be detected in mitochondria isolated from a hepatoma (47). If one considers the volume of a single liver mitochondrion and its nitrogen content, as estimated from mitochondrial counts (cf. 104), the concentration of soluble proteins within the structure must indeed be high.

As described in previous publications, the behavior of mitochondria in solutions of various tonicities and the lysis that occurs in hypotonic media are typical of an osmotically active system protected from its environment by a semipermeable membrane (52, 99). Recent experiments conducted in our laboratory have shed some further light on the properties of the mitochondrial membrane. The injection of fluoroacetate into female rats results in an accumulation in the liver of relatively large amounts of citric acid (14, 86). As indicated by the data in Table 5, most of the citric acid is recovered in mitochondria isolated and washed in solutions of sucrose. If the particles are washed with water instead of sucrose solutions, however, all their citric acid is extracted.[11] In addition to providing *in vivo* evidence for the mitochondrial localization of citric acid synthesis, this finding bolsters our confidence in the media commonly employed in the isolation of mitochondria. Thus, if the membranes of the particles are able to

[9] Although this procedure makes it possible to cover a large amount of territory in a relatively short time, its soundness as a cytochemical technic is open to serious question. A case in point is "citric oxidase," which, on the basis of studies of cyclophorase, is said to be a mitochondrial system (32). According to classical enzymology, the oxidation of citrate proceeds initially through the following enzymes: aconitase, isocitric dehydrogenase, TPN-cytochrome c reductase, and cytochrome oxidase. Although aconitase and isocitric dehydrogenase are largely recoverd in the soluble fraction of the cell, they are such powerful enzymes that their presence in trace amounts in the particulate fraction, perhaps by way of adsorption, would permit measurable oxygen uptake when citrate is added as a substrate. A situation of this type has, in fact, been demonstrated to hold in the oxidation of isocitrate (46).

[10] Lang *et al.* (65), using a viscosimetric method of enzyme assay, have reported that the desoxyribonuclease of kidney is mainly recovered in the nuclear fraction. The determination of the desoxyribonuclease activity concentrated in liver mitochondria (101) was based on the rate of release from highly polymerized DNA of ultraviolet-absorbing fragments soluble in dilute perchloric acid. The possibility must therefore be borne in mind that successive steps in the depolymerization of DNA may be carried out by more than one type of cell structure.

[11] W. C. Schneider, M. J. Striebich, and G. H. Hogeboom, unpublished experiments.

retain citric acid, it seems doubtful that appreciable quantities of soluble enzymes could be extracted during the isolation procedure. Bartley and Davies (4) have recently reported observations somewhat along the present lines. Thus, when kidney mitochondria were maintained under conditions of active metabolism, they were found to contain hydrogen, sodium, potassium, magnesium, and phosphate ions in concentrations 2–26 times as great as the corresponding concentrations in the surrounding fluid. The implications of these experiments in terms of renal physiology are, of course, of great interest.

The mitochondrial membrane is apparently also relatively impermeable to other compounds. If the particles are exposed to water or are mechanically disrupted, the activities of several enzymes, including acid phosphatase (8, 9, 29), glutamic dehydrogenase (51), and, as indicated previously, ATPase, are greatly enhanced. The same phenomenon was found to occur to a lesser extent in a study of the nucleases (101). With the exception of ATPase, all these enzymes pass into solution in the surrounding medium when the mitochondrial membrane is damaged. When the particle is intact, however, the membrane evidently forms a barrier between the enzymes and their substrates.[12] Cytochrome oxidase, on the other hand, and certain other enzyme systems whose substrates would not be expected to penetrate readily a semipermeable membrane seem to function at a maximal rate when the particles are intact. It is possible that the latter enzymes are located at or near the surface of the mitochondrion, whereas such enzymes as glutamic dehydrogenase and acid phosphatase are apparently present in a diffusible form inside the structure.

In addition to ATPase, several enzyme systems do not go into solution after disruption of mitochondria but remain firmly attached to fragments sedimentable in high speed centrifugation. Included are succinoxidase, DPN-cytochrome c reductase, cytochrome oxidase, and, interestingly, cytochrome c (45, 48). Fractionation of these disintegrated preparations at high speed in an ultracentrifuge has shown that DPN-cytochrome c reductase is associated with slowly sedimenting particles, whereas cytochrome oxidase and cytochrome c are found in a more rapidly sedimenting fraction (48). In these experiments it was also found that certain complex enzyme systems probably depend for their functioning on the structural integrity of the mitochondria. Thus, the succinoxidase system is partially inactivated by disruption of the particles, and octanoate oxidation is completely inactivated (45).

It is evident from this discussion that the biochemistry of the mitochondrion is certainly no less complex than its internal structure (84, 107), which was demonstrated so beautifully in the electron micrographs shown by Dr. Porter and Dr. Palade. Our present knowledge of the properties of liver mitochondria, including the existence of a semipermeable membrane and the presence of enzymes both in the soluble state and attached to the structural framework of the particle, provides a clue as to how reactions are controlled within the mitochondrion and integrated with reactions carried out elsewhere in the cell. Furthermore, there is an increasing body of evidence indicating that the morphological similarities of mitochondria of different tissues (84) are paralleled by similarities in biochemical properties (12, 13, 34, 35, 85, 94, 103). This observation has, in fact, been extended to include even the mitochondria of amoebae, and of insect, plant, and amphibian tissues (2, 10, 99, 118). In general, we feel that studies of the biochemistry of this structure have provided an impetus and a reasonably good beginning toward an understanding of the biochemistry of the cell as an integrated unit.

The Microsome Fraction

Approximately 10 years ago, Claude (19) described the isolation of particulate material from tissue extracts by prolonged centrifugation at gravitational forces of the order of 20,000. Although this cell fraction was at first thought to represent mitochondria, it was soon found to consist of particles too small to be resolved by the light microscope and is now referred to under the terms, microsomes or submicroscopic particles. The microsomes account for a considerable proportion of the mass or total nitrogen of the liver cell, some 20–25 per cent in the case of rat and mouse liver. It was recognized fairly early in semiquantitative analyses of the fraction that it contained rather high concentrations of pentosenucleic acid and phospholipid (21), but additional information concerning its biochemical properties was not obtained until recently and is still rather meager.

Table 6 lists those properties of the fraction that have been found in a concentration exceeding that in the homogenate. The list is short, and until Hers and collaborators (38) described their work on the specific glucose-6-phosphatase of liver, none

[12] In our opinion, the implication of this finding with respect to the validity of enzyme assays is of such importance that it is now imperative to determine routinely the effect on enzyme activities of disruption of the surface membranes of particulate structures (cf. 45, 48, 50, 51, 53, 100, 101).

of the properties studied appeared to be confined exclusively to microsomes. Thus, the cytochrome c reductases and uricase are also concentrated in mitochondria, and pentosenucleic acid and esterase are diffusely distributed in all other fractions; DPN nucleosidase is also found in nuclei and the soluble fraction. The recovery of glucose-6-phosphatase in the microsomes is, however, nearly complete, the small amount of activity remaining in the other fractions being readily explained on the basis of contamination by microsomes. A similar situation appears to hold for kidney lactonase, as reported by Meister (74).

Although complete studies of the distribution of lipids in liver homogenates have not, to our knowledge, been reported, investigations of individual fractions have indicated that the microsomes contain much higher concentrations of lipid than does any other cell fraction. Apparently, about 40 per cent of the dry weight of microsomes is comprised of lipid, which is mainly in the form of phospholipid (cf. 99). The fact that liver microsomes are pigmented, showing a distinctly reddish hue, has also interested a number of investigators. Strittmatter and Ball (114) have presented evidence indicating that the pigment is a protoporphyrin hemochromagen resembling but nevertheless distinct from cytochromes a, b, and c.

Obviously, it is not at this time possible to present anything like a complete picture of the role of microsomes in cellular metabolism. Except for their apparent ability to transfer electrons between cytochrome c and the pyridine nucleotides, the particles evidently do not play an important part in oxidative or respiratory metabolism. The high PNA content of the fraction and an apparent relationship between PNA and protein synthesis have, however, led to some speculation concerning the possible role of microsomes in the synthesis of proteins. In this respect, more recent *in vivo* experiments have shown that, after injection of labeled amino acids, the proteins of microsomes show a higher specific activity than do the proteins of any other fraction isolated from liver (55, 66). Siekevitz (106) has extended these observations by showing that when respiring liver homogenates are incubated in the presence of alanine-C^{14}, both the rate of incorporation and the total incorporation of the amino acid are much higher in microsomes subsequently isolated from the reaction mixture than in any other cell fraction. He has also shown that, whereas incorporation does not occur when microsomes or mitochondria are incubated separately with the substrate, incorporation does occur when the two fractions are combined. A requirement for the reaction is the addition of substrates and co-factors permitting the simultaneous occurrence of oxidative phosphorylation. During this oxidative reaction, the mitochondria apparently produce a soluble factor, possibly a derivative of ATP, that enables the microsomes to incorporate alanine. These very interesting experiments thus suggest that microsomes contain the enzyme system responsible for the incorporation of the amino acid but in order to carry out the reaction require a source of energy which in this case is provided by the respiratory enzyme systems of the mitochondrion. These findings may well represent another example of the integrated activities of several cell structures in carrying out a complicated metabolic process. There are, in addition, data indicating that microsomes play some role in

TABLE 6

BIOCHEMICAL PROPERTIES OF MICROSOME FRACTION

Enzyme system or compound	Concentration (whole tissue = 1)	Recovery (whole tissue = 100)	Reference	Remarks
Esterase	4.2	47	(82)	Diffusely distributed in all other fractions.
DPN-cytochrome c reductase	2.5	59	(44)	Also concentrated in mitochondria (cf. 41).
TPN-cytochrome c reductase	2.0	36	(46)	Also concentrated in mitochondria.
Glucose-6-phosphatase	?	88	(38)	
Lactonase (kidney)	3.7	71	(74)	
DPN nucleosidase	?	50(?)	(115)	Over-all recovery 130–140 per cent; see Table 2.
Uricase	1.2	32	(100)	See Table 4.
Pentose nucleic acid	2.3	50	(99)	Present in all other fractions.
Phospholipid	?	?	(99)	Data incomplete (cf. 3).

Krebs cycle reactions (103), perhaps through their content of the cytochrome reductases, and in glycolysis (68).

An important question relates to the intracellular structures represented by the isolated microsome fraction. From time to time the microsomes have been considered to be artifacts derived from other cell structures. Claude's initial suggestion that the fraction represents the basophilic ground substance of the cell has, however, received considerable experimental support (11). In addition, numerous studies with the electron microscope have clearly shown that the cell contains an abundance of particulate structures that are too small to be seen in the light microscope and would be expected to sediment at centrifugal forces of the order required for the sedimentation of microsomes. Palade, in fact, has found that the microsome fraction isolated from liver homogenates is largely composed of structural elements that are morphologically similar in the electron microscope to the endoplasmic reticulum.[13]

There is, furthermore, a considerable amount of biochemical evidence indicating that microsomes are a group of distinct cellular entities. In this respect, Green's conclusion (32) that they may be fragments of mitochondria is not supported by data obtained in a number of other laboratories. There is good reason to believe, in fact, that qualitative differences exist between microsomes and mitochondria. Thus, when adequate fractionation of cytoplasmic elements is achieved, glucose-6-phosphatase would appear to reside exclusively in microsomes (38). Conversely, the specific cytochrome oxidase activity of the mitochondrial fraction is almost 20 times that of the microsomes (98), and the low activity of the latter fraction can be readily explained on the basis of the presence of contaminating mitochondria. In addition, mitochondrial fragments, although similar in sedimentation characteristics to microsomes, are qualitatively different in their biochemical properties. Thus, when isolated mitochondria are disrupted by means of sonic vibrations to yield optically empty suspensions, the mitochondrial fragments isolated by high speed centrifugation show an even higher specific cytochrome oxidase activity than do the original preparations of intact mitochondria (45). It seems possible that Green's inability to distinguish biochemically between microsomes and mitochondria (32) may result from his use of the Waring Blendor in the preparation of homogenates. Since it is well known that this method of homogenization results in the disruption of a significant proportion of the mitochondria (42, 99), the microsome fraction isolated from such homogenates would be expected to contain mitochondrial fragments and thus to exhibit the enzyme activities characteristic of the latter particles.

[13] G. E. Palade, personal communication.

It should not be inferred from this discussion that we believe the microsome fraction to be homogeneous and thus to represent a single cellular entity. There is, in fact, plenty of evidence to the contrary. Chantrenne's observations (16) on the biochemical heterogeneity of these particles have been confirmed and extended by Barnum and Huseby (3) and, more recently, by Novikoff and his collaborators (81). The original implication of Chantrenne's work, that *all* cytoplasmic particles form a continuous spectrum in regard to both size and biochemical properties, has not, however, been supported by subsequent work. There would appear to be a distinct hiatus between mitochondria and microsomes both biochemically, as just discussed, and with respect to rate of sedimentation. The latter situation can be readily demonstrated in the analytical centrifuge if a homogenate is freed of nuclei and the resulting opaque suspension of cytoplasmic elements centrifuged at low speed.[14] The mitochondria, which account for the opacity of the suspension because they are large enough to scatter light, are completely cleared long before sedimentation of microsomes occurs. The latter elements give a refractive index pattern that is diffuse and shows several peaks, thus attesting to the presence of particles of several ranges of size or density.

The Soluble Fraction

After the removal from the liver homogenate of nuclei, mitochondria, and microsomes, the latter by 1 hour's centrifugation at about $60{,}000 \times g$, there remains a supernatant accounting for about 40 per cent of the original total nitrogen. Most of the nitrogen of this "soluble fraction" of the cell is in the form of proteins in solution. In addition, lipids are present that migrate centripetally in the centrifuge, but there is apparently little other material that is not in true solution. Although we have assumed for operational purposes that the soluble fraction represents the "cell sap," we must also recognize the possibility that it may include substances extracted from the particulate elements of the cell. The latter consideration imparts a definite risk to any unequivocal conclusions concerning the cytochemical significance of biochemical analyses of this supernatant and, we must admit, detracts somewhat from our interest

[14] G. H. Hogeboom and W. C. Schneider, unpublished experiments.

in the fraction. Nevertheless, in view of evidence indicating that soluble proteins are *not* extracted under proper conditions from such structures as nuclei and mitochondria, it is our opinion that analyses of the soluble fraction are in many instances significant from the cytochemical standpoint. As in studies of the biochemical properties of the other isolated cell constituents, however, all conclusions must be carefully weighed in terms of the concentration and recovery obtained.

The presence in the supernatant of a large number of proteins is demonstrated by the complicated patterns obtained in electrophoretic studies by Sorof and Cohen (109, 110) and Gjessing, *et al.* (31). That a correspondingly large number of enzymes should have been found in the fraction is not, of course, surprising. Since time does not permit a detailed discussion of the biochemical properties of the supernatant, we shall restrict our comments to a few findings relating to reactions that are of major importance in cellular metabolism.

Perhaps the best example is the role of the soluble fraction in anaerobic glycolysis, i.e., the series of reactions converting glucose or glycogen to lactic acid. LePage and Schneider (68), in a study of the rate of lactic acid formation in an anaerobic system including glucose, hexosediphosphate, pyruvate, ATP, and DPN, found that over 50 per cent of the activity of rabbit liver homogenates could be recovered in the soluble fraction. The other cell fractions showed little or no activity by themselves but were capable of stimulating the activity of the supernatant. Although the complexity of the reaction is such that the rate-limiting component is not known, the implication of this investigation is that the supernatant contains most of the enzymes involved. The enhancement of lactic acid formation by the particulate fractions is not readily explained but probably comprises another example of the integrated activity of several cell structures in carrying out a complicated metabolic process. A complete picture of the intracellular locale of glycolytic enzymes will be available, however, only when studies are made of the distribution among cell fractions of each individual enzyme. Some recent steps in this direction all lend support to the view that glycolysis is largely a function of the soluble fraction of the cell. Thus, Hers, de Duve, and their collaborators (30, 38) have shown that hexokinase, phosphorylase, and phosphoglucomutase are mainly recovered in this fraction. A similar situation has been found in studies of the intracellular distribution of lactic dehydrogenase[14] and of aldolase (56).

As indicated earlier in this discussion, the soluble fraction, through its content of isocitric dehydrogenase (46) and aconitase,[8] also takes part in the reactions of the Krebs cycle. The presence in the fraction of 90 per cent or more of the adenosine deaminase and nucleoside phosphorylase activity of liver homogenates (100) demonstrates a probable role in the metabolism of purines. The occurrence of certain other enzymes and compounds (18, 62, 73, 99), as well as the numerous instances in which the supernatant has been used as the starting material in the purification of enzymes, attests further to the biochemical complexity of the fraction.

SUMMARY AND CONCLUSIONS

Despite several areas of confusion and doubt, mostly attributable to defective methods, the cytochemical data presented here permit us to arrive at certain general conclusions that are difficult to refute. Thus, it is obvious that, from a biochemical standpoint, the cell is not simply a bag of enzymes arranged in a haphazard fashion and dependent only on the laws of diffusion for their integrated function. Nor is the cell biochemically only a nucleus or, as some schools of thought would apparently have it, a collection of mitochondria. It is, on the other hand, a complicated mosaic of structural units that are endowed with specific biochemical properties. Furthermore, although some degree of functional autonomy may be indicated in the case of certain elements, the mitochondria in particular, there is reason to believe that in no single instance is this autonomy complete but rather that the structural units are mutually dependent on one another in their contribution to the metabolism of the cell. The data would also appear to have some bearing on differentiation, or at least that aspect of differentiation involving the strategic intracellular location of these specifically endowed structures so that their individual activities can be integrated efficiently in the over-all function of a tissue. In heart muscle, for example, the relationship of the mitochondrion to the myofibril (36, 84) is an ideal arrangement for the resynthesis of ATP degraded during muscular activity. In view of the experi nents of Bartley and Davies (4) on the secretory activity of kidney mitochondria, the polar distribution of these structures in the tubular cells of the kidney would seem to be another example. These are not new ideas. For many years, the cytologist has had them in mind. Now, however, there are concrete biochemical data to back them.

In a consideration of the future of cytochemistry, the important questions are what, specifically and incontrovertibly, is the function of each type

of cell structure, and, to be less academic anp more practical, what happens to these structures in cellular dysfunction. It is reasonable to predict, for example, that cytochemical investigations will eventually play an important role in the understanding of carcinogenesis. Although some cell fractionation studies have been concerned with unraveling the carcinogenic process, the cytochemical information available in that field is much less complete than that available for the normal cell. There is still a great deal to be done before the problem of cancer can be discussed intelligently from the cytochemical standpoint.

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The Effect of Roentgen Radiation on the Incorporation of Radiophosphorus into Nucleic Acids and Other Constituents of Mouse Mammary Carcinoma*

HALVOR VERMUND,† CYRUS P. BARNUM, ROBERT A. HUSEBY,‡ AND KARL W. STENSTROM

(Departments of Radiology, Physiological Chemistry, and Physiology, Medical School, University of Minnesota, Minneapolis, Minn.)

A number of investigators have studied the effect of ionizing radiations on the incorporation of isotopically labeled substances into various cellular constituents (1–3, 9–14, 16, 18–20). An evaluation of the present status is rendered difficult because of differences in the manner of administering the radiation, i.e., whole-body or local irradiation, because of questions concerning the isotopic purity of the substances isolated and because some conclusions have been drawn on the basis of observed differences that are not statistically significant. This report is confined to a consideration of the effect of roentgen radiation on the incorporation of P^{32} into various organic compounds of transplanted mouse mammary carcinomas. The irradiation has been limited to a leg bearing the transplanted carcinoma; we believe the substances isolated are sufficiently pure to give real meaning to the results; and the differences have been analyzed for statistical significance.

MATERIALS AND METHODS

Several spontaneous mammary carcinomas arising in Z(C3H) and AZF_1 hybrid mice were transplanted over several generations into inbred mice of 6–8 weeks of age possessing the necessary genetic constitution for progressive growth of the tumors. ZBC mice for the most part were used for carrying the Z tumors and AZF_1 or the corresponding strain without the milk agent ($A_xZ_bF_1$) for the AZF_1 tumors.[1] Transplantations were made by injecting 0.1 ml. of a 10–20 per cent tumor cell suspension into the subcutaneous layers of the dorsal aspect of both hind legs. After the first few transfers the tumors grew in nearly all the animals injected. When the tumors had reached a size of approximately 1–1.5 cm. in outside diameter after a period of 2–3 weeks, the right leg up to the groin, including the tumor transplant, was exposed to the roentgen rays, while the rest of the animal including the tumor-transplant on the left leg was shielded in a specially constructed lead protection device (20).[2]

Histology.—The tumors had the histological structure of an adenocarcinoma with sparse connective tissue stroma and large hyperchromatic cell nuclei with a high number of mitotic figures.

One tumor which grew slowly had more abundant connective tissue stroma and showed a consistently lower uptake of P^{32} into the DNA than the other tumors.

Radiation factors.—A General Electric x-ray therapy machine was operated at 4 m. amp. and 140 kv. with a filter of 2 mm. Al, giving an HVL of 0.17 mm. Cu (effective wavelength 0.30 Å). In the first experiments a distance of 30 cm. between the target of the x-ray tube and the tumors was used, giving an output of 49 r.p.m. when measured in air with a Victoreen ionization chamber. An increase of 10 per cent to this dose was contributed by backscatter at the position of the tumors when the animals were mounted in the protection device. In most experiments a total dose of 4,500 r in air was delivered to the right leg over a period of approximately 92 minutes. In the later experiments the arrangement was changed to 5 ma. and a focal-tumor distance of 20 cm. with an output of 115 r.p.m. in air. In a few experiments doses of 450 r or 9,000 r were administered.

Ionization chambers placed in close approximation to the shielded tumors on the left leg gave readings which varied around 0.1 per cent of the dose delivered to the right leg (20). This small amount was due to scattering of the x-rays and could not be eliminated. A total of ten animals could be irradiated at one time in a homogeneous field. No anesthesia was used. The right foot was kept in place by clamping the distal end with a small battery clamp attached to a thin copper wire. By this arrangement the right leg up to the groin was immobilized during the period of exposure while

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† For part of the time a Fellow of the Norwegian Cancer Society and later a Fellow of The James Picker Foundation for Radiological Research.

‡ Present address: Dept. of Surgery, University of Colorado Medical School, Denver.

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otherwise the animal was allowed to move on the outside of the lead cylinder.

The radioactive tracer.—P^{32} was injected intraperitoneally in doses varying from 0.5 to 1 μc/gm animal body weight in a volume of approximately 0.5 ml. of 0.85 per cent sodium chloride.[3, 4] In some experiments the injection was made immediately following the end of the irradiation, while in others a time interval of 4, 12, or 24 hours elapsed between the end of the exposure to x-rays and the injection of P^{32}. In all experiments at exactly 1 hour following the injection of P^{32} the animals were sacrificed, and the tumors rapidly dissected.

Preparation of the tissues.—Tumors from four to eight animals were pooled, chilled in ice-baths, and passed through a tissue-press. Tumors from the right irradiated and the left shielded leg were kept separate, and tumors from nonirradiated animals of the same litter, inoculated with the same tumor cell suspension, were used as controls.

The pressed tissue was homogenized and fractionated as previously described (5, 7). The fractions obtained for study were the nuclei, microsomes, ultrasedimentable particles (100,000 $\times$ *g* for 60 minutes), and the nonsedimentable supernatant fluid. From each of these fractions the nucleic acids, phospholipides, and "phosphoproteins" were extracted and purified (6, 7, 15). The purified organic compounds and the inorganic phosphorus obtained from an aliquot of the original homogenate were wet-ashed and analyzed for P^{32} and total P (6).

As previously reported (7), it is possible to prepare a relatively pure PNA (pentosenucleic acid) from liver nuclei by extraction of the lipide-free nuclei in a cold buffer at pH 10. This PNA accounts for about 50 per cent of the PNA present in the lipide-free liver nuclei and, when isolated after administration of P^{32} to the animals, has a specific activity comparable to the remaining half that sediments along with the DNA (desoxypentosenucleic acid) in the pH 10 buffer. In nuclei isolated from mouse mammary carcinoma, on the other hand, the PNA extracted by the cold pH 10 buffer (fraction I) amounts to only about 20 per cent of the total present and has a specific activity significantly below that of the remaining 80 per cent (fraction II). Fraction II has been prepared by extracting the pH 10 sediment with 6 per cent NaCl in pH 10 buffer for 30 minutes in a boiling water bath. Treatment of this extract with 2 volumes of 95 per cent alcohol precipitates the DNA and leaves the PNA in the alcoholic supernatant fluid. The alcohol is evaporated off, and the PNA extracted from the residue with cold 5 per cent TCA (trichloroacetic acid).

Modification of extraction method for DNA.—In experiments designed to study only the incorporation of P^{32} into DNA, the need for a rapid method of extracting and purifying the DNA became apparent. Several existing methods were tested, and the following adaptation evolved, because it gave good yields of highly purified DNA in the shortest time. The nuclear fraction was washed several times with cold 2 per cent citric acid, then extracted with pH 10-buffered 6 per cent NaCl for 30 minutes in a boiling water bath. The residue was re-extracted twice with fresh buffered NaCl and the extracts pooled and treated with 2 volumes of 95 per cent alcohol and left at room temperature at least 1 hour. The precipitate was re-extracted, this time with 10 per cent NaCl, leaving behind some contaminating protein that had been carried along on the original extraction, and the extract again treated with 2 volumes of alcohol. The resulting precipitate was heated with a small volume of 0.1 N NaOH at 80° C. for 20 minutes to hydrolyze any remaining traces of PNA, then cooled, made about 0.1 N to HCl and 5 per cent with respect to TCA, and allowed to stand 1 hour in an ice-water bath. The resulting precipitate was washed thoroughly with cold 5 per cent TCA and then heated with 5 per cent TCA at 80° C. for 30 minutes to solubilize the DNA and leave behind any small amounts of protein that might still contaminate the preparation.

Criteria for purity.—Since the specific activity of P varies considerably from one organic compound to another, it is essential to obtain the compounds in the highest state of purity. In establishing the methods the criteria for purity have been those previously described (6, 7, 15). In the case of DNA the mean N/P ratio was found to be 1.68 with a standard error of 0.013 in 24 consecutive analyses, and colorimetric analyses (7) indicated little, if any, contamination with PNA. Furthermore, additional extractions of DNA with 6 per cent NaCl-pH 10 buffer and reprecipitation with alcohol failed to alter the specific activity of the DNA. In the case of PNA, isolated by these procedures from mouse mammary carcinoma, that from the cytoplasmic particulates has shown an N/P ratio of 1.75 to 1.79, that from the cytoplasmic supernatant fluid 2.35, and that from the nuclei (fraction II) as high as 4 or 5. Despite the high N/P ratios in some of these fractions, the pentose content based on the orcinol-HCl reaction (measuring purine-bound pentose only) has ranged from 49.5 to 52.5 per cent of that calculated from the P content. It has been concluded, therefore, that the bulk, if not all, of the P is PNA-P.

Calculations.—Standard solutions of the radioactive material injected were always made up by dilution in 0.1 N H_2SO_4 and counted with the samples. The specific activity of each sample was calculated (17) as the biological concentration coefficient (BCC) as follows:

$$\text{BCC} = \frac{(\text{counts/min/mM P}) \times 100}{\text{counts/min injected/gm body weight}}.$$

Most values reported in this paper refer to the relative specific activity (RSA) which is defined as follows:

$$\text{RSA} = \frac{\text{BCC of organic compound}}{\text{BCC of tumor inorganic P}} \times 100.$$

This relative specific activity served as the basis for comparison between the different samples to evaluate the effect of irradiation on the incorporation of P^{32} into the organic compounds.

RESULTS

Effect of x-radiation on the incorporation of P^{32} into DNA.—Table 1 shows all the observations made on DNA from tumors in control mice and in mice in which the tumor on one leg was irradiated with 4,500 or 9,000 r. We have not observed any statistically significant difference between these dose levels. It may be seen that the mean incorporation of P^{32} into the DNA of the irradiated tumor is 53 per cent of that for the controls and that this is a highly significant inhibition. Chart 1 shows the rather large variations encountered in these measurements and emphasizes the necessity of demonstrating the statistical significance of observed differences. These data refer to experiments in which the P^{32} was injected at various time intervals between zero and 24 hours after irradiation. In Table 2, on the other hand, all the data

[3] P^{32} was supplied as Na_2HPO_4 on allocation from the Oak Ridge National Laboratory.

[4] Trial experiments indicated that the relative specific activity of the tumor DNA, 1 hour after administration of P^{32}, was not significantly affected by the dose given in the range from 0.1 to 10 μc/gm body weight.

with respect to animals receiving 4,500 r in which directly irradiated and shielded tumors were compared to nonirradiated controls are broken down into the various time intervals that were investigated. Here the marked and significant inhibition in the irradiated tumor is also apparent, but, in addition, there appears to be a less marked but relatively significant inhibition in the shielded tumor 4 hours after irradiation. It is possible that a similar inhibition in the shielded tumor occurs immediately following irradiation, but the data are not sufficient to demonstrate the statistical significance of this inhibition.

TABLE 1

EFFECT OF IRRADIATION ON RELATIVE SPECIFIC ACTIVITY OF DNA*

Summary of all experiments with 4,500 or 9,000 r

	No. experiments	Mean RSA±S.E.†	Significance of difference between means (=P)‡	
Controls (C)	68	0.85±0.05	C and S	0.23
Shielded (S)§	53	0.78±0.04	C and I	<0.0001
Irradiated (I)	53	0.45±0.03	S and I	<0.0001

* In all experiments reported in this paper the time between injection of P^{32} and sacrifice of the animals has been 1 hour.

† Standard error (S.E.) $= \sqrt{\frac{\overline{X^2}}{N} - \overline{X}^2} \cdot \frac{1}{\sqrt{N}}$ where X is the individual value of one experiment, $\overline{X}$ the mean of all experiments, and N the number of separate experiments.

‡ P is the probability that "Student" t will be exceeded or that the observed difference could have occurred by chance.

§ The observations on shielded tumors are included here for comparative purposes only, since, as may be seen in Table 2, the RSA in this group appears to vary depending upon the time between irradiation and injection of P^{32}.

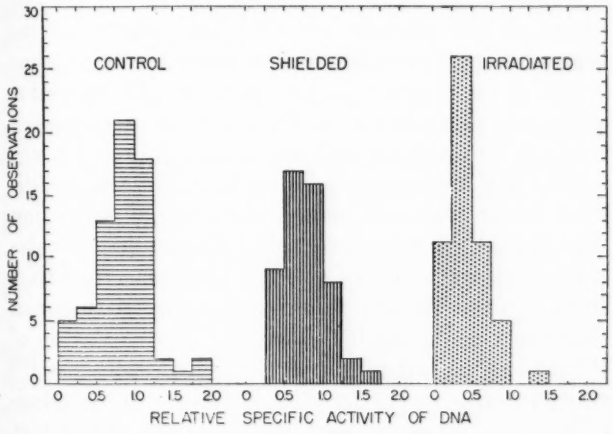

CHART 1.—The frequency distribution of the relative specific activity of DNA isolated from tumors in nonirradiated control animals and from shielded and directly irradiated tumors in animals receiving 4,500 or 9,000 r of local irradiation. P^{32} injected at intervals varying between 0 and 24 hours after irradiation. All animals sacrificed 1 hour after P^{32} injection.

In eleven experiments in which only 450 r was administered, the mean value (RSA) for DNA from the irradiated tumor was 67 per cent of that of the controls, but this difference has only borderline statistical significance with a P value of 0.04. No inhibition was seen in the shielded tumors in these animals receiving 450 r. Because of the small amount of scattered radiation unavoidably received by mice despite the lead protection device, four experiments were set up in which mice bearing tumors were given 20 r of whole-body radiation. This dose was calculated as the maximum of scattered radiation that might have been received by the shielded part of the body. No inhibition of P^{32} incorporation into the tumor DNA was seen in these animals. In another group of four experiments we failed to find any inhibition in a tumor when a normal leg was irradiated with 4,500 r while the rest of the animal including the tumor was shielded.

TABLE 2

RELATIVE SPECIFIC ACTIVITY OF DNA AT DIFFERENT TIME INTERVALS FOLLOWING IRRADIATION WITH 4,500 r

	Time between irradiation and P^{32} injection (hours)	No. experiments	Mean RSA ±S.E.	Significance of difference between means (=P)	
C		5	1.19±0.20	C and S	>0.5
S	0	5	0.97±0.22	C and I	0.007
I		5	0.46±0.11	S and I	0.02
C		15	0.88±0.13	C and S	0.02
S	4	15	0.72±0.09	C and I	<0.001
I		15	0.45±0.06	S and I	<0.001
C		3	0.79±0.08	C and S	>0.5
S	12	3	0.81±0.09	C and I	0.04
I		3	0.32±0.18	S and I	0.04
C		9	0.97±0.17	C and S	>0.5
S	24	9	0.94±0.12	C and I	0.007
I		9	0.41±0.08	S and I	0.002

C = control; S = shielded; I = irradiated.

Failure to demonstrate an effect of x-radiation on the incorporation of P^{32} into PNA, phospholipides, or "phosphoproteins."—In Table 3 are summarized our attempts to demonstrate an effect of x-rays on PNA metabolism. It may be seen that in none of the PNA fractions obtained from nuclei or cytoplasm is there a statistically significant difference between irradiated and control tissues. Only for fraction I of the nuclear PNA from animals injected with P^{32} immediately after irradiation did any difference approach statistical significance. In this instance the mean of six experiments showed PNA from irradiated tumors with a RSA 66 per cent of that of PNA from shielded tumors. The significance of the difference between the means was 0.065.

Table 4 summarizes our inability to demonstrate any effect of x-rays on the incorporation of

P^{32} into either phospholipides or "phosphoproteins." In the case of these two constituents the specific activities, 1 hour after administration of P^{32}, do not vary greatly from one cellular fraction to another. Since no statistically significant differences were observed between irradiated and control animals for any of the fractions, we have pooled the data.

TABLE 3

RELATIVE SPECIFIC ACTIVITY OF PNA FOLLOWING IRRADIATION WITH 4,500 r*

Sample	No. experiments	Mean RSA±S.E.	Significance of difference between means (=P)	
Nuclear fraction I:				
C	11	6.35±0.66	C and S	0.5
S	16	5.86±0.52	C and I	0.5
I	16	5.00±0.72	S and I	0.33
Nuclear fraction II:				
C	15	18.2±3.4	C and I	0.5
S	16	18.2±2.8	S and I	0.5
I	16	16.7±2.4		
Microsomes:				
C	13	0.65±0.06	C and S	0.5
S	16	0.58±0.07	C and I	0.15
I	16	0.53±0.06		
Ultra-sedimentable particles:				
C	12	0.57±0.11	C and S	0.5
S	10	0.63±0.10	C and I	0.5
I	10	0.66±0.14	S and I	0.5
Supernatant fluid:				
C	13	1.30±0.23	C and S	0.5
S	10	1.67±0.20	C and I	0.5
I	10	1.49±0.26	S and I	0.5

* Summary of all experiments in which P^{32} was injected between 0 and 24 hours following irradiation. All animals sacrificed 1 hour after P^{32} injection.
C = control; S = shielded; I = irradiated.

TABLE 4

RELATIVE SPECIFIC ACTIVITY OF PHOSPHOLIPIDES AND "PHOSPHOPROTEINS" FOLLOWING IRRADIATION WITH 4,500 r*

Sample	No. experiments	Mean RSA±S.E.	Significance of difference between means (=P)	
Phospholipides:				
C	40	2.4±0.18	C and S	>0.5
S	45	2.8±0.23	C and I	>0.5
I	45	2.8±0.21		
"Phosphoproteins":				
C	25	21.3±1.4	C and S	>0.5
S	27	23.2±1.7	C and I	>0.5
I	27	23.5±2.3	S and I	>0.5

* Values are means from all cellular fractions and from all experiments in which P^{32} was injected between 0 and 24 hours following irradiation. All animals were sacrificed 1 hour after P^{32} injection.
C = control; S = shielded; I = irradiated.

Effect of x-rays on the specific activity of inorganic phosphorus.—It may be seen in Table 5 that the specific activity of the tumor inorganic phosphorus is less in the irradiated tumors than in controls and that the difference has a high degree of statistical significance. In experiments in which control animals were immobilized in the shielding device over the same period of time we observed no similar decrease, suggesting that it is the x-radiation which is responsible for this effect. A point that is not clearly established is whether this decrease is proportional throughout the 1-hour period or whether there is a qualitative difference in the shape of the specific activity-time curve of inorganic phosphorus in the irradiated tissue as compared to the control. If, for example, the curve for inorganic phosphorus in the controls had passed through a maximum and was descending at 1 hour, while that for inorganic phosphorus of the irradiated tissue was still rising, then our observation that the DNA from irradiated tissues had a relative specific activity of about ½ that seen for DNA from control tissues would not necessarily mean that the x-radiation had inhibited incorporation of P^{32} into DNA but might merely mean that less P^{32} was available in the irradiated tissues. This possibility does not seem very likely in view of the fact that the relative specific activities of the PNA, phospholipide, and "phosphoprotein" are unaffected by the irradiation. If, in fact, the rates of turnover of these constituents are unaffected by the x-rays, then their specific activities at the end of the 1-hour period will be a certain function of the specific activity of inorganic phosphorus throughout the 1-hour period. In Table 6 are recorded a number of experiments in which the specific activities of the DNA and of the nuclear phospholipide were compared. It may be seen that, on this basis also, there is a selective inhibition of the incorporation of P^{32} into the DNA by the irradiation. Also noticeable in this group of experiments is a more pronounced inhibition in the shielded tumor at the 4-hour interval. This in-

TABLE 5

EFFECT OF IRRADIATION ON THE BIOLOGICAL CONCENTRATION COEFFICIENT (BCC) OF INORGANIC PHOSPHATE FROM MAMMARY CARCINOMA

Summary of all experiments with 4,500 or 9,000 r

Sample	No. experiments	Mean BCC±S.E.	Significance of difference between means (=P)	
C	68	7,027±265	C and S	>0.5
S	53	6,949±256	C and I	0.0005
I	53	5,683±283	S and I	0.001

C = control; S = shielded; I = irradiated.

direct effect on the shielded tumor has been difficult to produce consistently but has appeared more frequently in some transplant lines than in others.

TABLE 6

COMPARISON OF SPECIFIC ACTIVITIES OF DNA AND NUCLEAR PHOSPHOLIPIDE IN THE SAME TISSUE SAMPLE FOLLOWING IRRADIATION WITH 4,500 r

			0-HOUR INTERVAL†		4-HOUR INTERVAL†	
SAMPLE	No. exp.	MEAN RATIO* ±S.E.	No. exp.	Mean ratio ±S.E.	No. exp.	Mean ratio ±S.E.
C	12	55.6±5.2				
S			6	44.2±4.8	5	27.8±3.5
I			6	21.0±3.8	5	23.4±6.0

* Ratio = $\frac{\text{RSA of DNA} \times 100}{\text{RSA of nuclear phospholipide}}$.

† Time between end of irradiation and injection of P^{32}. All animals sacrificed 1 hour after P^{32} injection.
C = control; S = shielded; I = irradiated.

SUMMARY AND CONCLUSIONS

These observations confirm the findings of v. Euler, Hevesy, and Ahlström (2, 3, 4, 9, 10) and of Payne, Kelly, and Entenman (16) that x-radiation causes a marked and statistically significant inhibition of the incorporation of P^{32} into DNA. In extension of the work of v. Euler *et al.*, and in contradistinction to the contention of Holmes, based on the work with Jensen rat sarcoma (13, 14), our observations demonstrate that, in mouse mammary carcinoma, this inhibition remains statistically significant and undiminished for at least 24 hours after irradiation.

Our results on mouse mammary carcinoma do not show as marked or as consistent an inhibition of DNA metabolism in a shielded tumor as that reported for a shielded Jensen sarcoma in rats (3). Here the difference may lie in the manner of administering the roentgen radiation. It is also true from our experience that some transplant lines more consistently show this indirect effect than others.

Our observations cannot confirm the reports of Holmes (13, 14), of Thomson *et al.* (19), of Payne *et al.* (16), or of Abrams (1) that roentgen radiation affects PNA metabolism. We have not been able to demonstrate a statistically significant effect though it is possible that a more extensive study might do so. It is true that Abrams was studying the incorporation of glycine into nucleic acid purines, and it is possible that these reactions might be inhibited by x-radiation in the absence of any effect on P^{32} incorporation.

With respect to phospholipide metabolism, we find no inhibition of P^{32} incorporation from local irradiation of transplanted mammary tumors. These observations differ from those of Hevesy (11), who used total body irradiation and studied other types of tissue.

Much of the work so far reported, whether with carbon-labeled precursors (12, 18) or with P^{32} (3, 4, 9, 10), indicates that roentgen radiation inhibits DNA "synthesis" to approximately 50 per cent of that observed in a control tissue. Our observations demonstrate that 9,000 r causes no significantly greater inhibition than seen with 4,500 r or than seen with 1,000–2,000 r total-body radiation administered by other workers. Furthermore, 4,500 r causes a complete cessation of tumor growth for at least 2 weeks (20) and prevents any new cells from entering mitosis.[5] These facts augment the suggestion made by v. Euler and Hevesy that the residual P^{32} uptake by DNA after x-radiation is not due to new formation of DNA in cells not yet affected by the radiation but rather to "turnover" of preexisting DNA. The fact that DNA in adult liver cells incorporates extremely little P^{32} (6) and that P^{32} once incorporated into DNA during liver regeneration is tenaciously held (8) would seem to preclude such "turnover" from occurring in resting cells. This leads to the suggestion that the residual incorporation of approximately 50 per cent, seen after irradiation has caused a cessation of growth, may be ascribed to a "turnover" of DNA in those cells that are preparing to undergo division. The implication is that roentgen radiation blocks the synthesis of new DNA but does not block the stimuli responsible for preparing the cells for this new synthesis.

It bears emphasis again that, at least in these mouse mammary carcinomas, the effect of x-radiation is amazingly specific. It blocks steps leading to the formation of new DNA but is without demonstrable significant effect on the incorporation of P^{32} into PNA, phospholipides, or "phosphoproteins."

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Ascorbic Acid Analog in Experimental Cancer*

B. Sokoloff, W. H. Eddy, R. Powella, J. Beaumont, and H. Relos

(*Southern Bio-Research Laboratory, Florida Southern College, Lakeland, Fla.*)

In 1943, Woolley and Krampitz (31) postulated that 2,3-enediol-D-glucoheptono-1,4-lactone (D-glucoascorbic acid) was an antagonist of ascorbic acid, since rats and mice fed 10 per cent of the compound in a purified diet developed a scurvy-like syndrome. They noted diarrhea, extensive hemorrhage in the chest, leg, tail, and gingiva, and growth failure. They observed, however, that ascorbic acid did not reverse the effect of D-glucoascorbic acid, except in guinea pigs (29), and that a natural diet, containing certain factors present in liver, prevented the manifestations induced by D-glucoascorbic acid. Banerjee and Elvehjem (1), confirmed the observation that diarrhea and growth failure developed and found that these disturbances were prevented by incorporating in the diet 6 per cent of 1:20 liver extract. Gould (11) could find no antagonism between D-glucoascorbic acid and ascorbic acid as judged by histologic examination of the jaws of guinea pigs fed the analog or by estimation of the serum phosphatase levels. Gorlin (10) added 5 per cent of D-glucoascorbic acid to the diet of mice. He observed diarrhea, growth cessation, perianal inflammation, and alopecia but no subcutaneous hemorrhages, hematomas, or inflamed gingiva and, in general, no histologic evidence of scurvy. This was confirmed by Schafer (23), who used rats, hamsters, and guinea pigs. On the other hand, Lan and Sealock (14) and Painter and Zilva (18) showed that D-glucoascorbic acid exerted activity in the oxidation of tyrosine by liver homogenates similar to that of ascorbic acid as described by Sealock and Goodland (24). But LaDu and Greenberg (13) suggested that ascorbic acid may act in a less specific manner and showed that several other compounds, including hydroquinone, also increased tyrosine oxidation. According to Zilva (32), the analog is not retained in the body and has very little antiscorbutic activity. Sokoloff *et al.* (25) reported that the addition of 1–2 per cent D-glucoascorbic acid to a Sherman-LaMer diet brought the ascrobic acid concentrations of blood plasma, adrenal, liver, and spleen close to zero in rats and mice without inducing diarrhea, loss of weight, or hemorrhagia. In his recent review, Woolley (30, p. 39) stated that "so far as present evidence goes it seems to indicate that D-glucoascorbic acid, when added to a highly purified ration, causes in guinea pigs a disease which is not exactly like scurvy but has some similarities to it. . . . This substance is undeniably analogue in structure to ascorbic acid. . . . "

MATERIALS AND METHODS

The British brown-white strain of rats, 168-AW, originally obtained from the Crocker Cancer Laboratory of Columbia University, black mice, strain C57N, originally supplied by Jackson Memorial Laboratory, and guinea pigs of family 2 from the National Cancer Institute were used. Crocker (August) rat carcinoma, mouse adenocarcinoma E 0771, and liposarcoma of guinea pigs were used for testing the effect of D-glucoascorbic acid.

The Sherman-LaMer scorbutogenic diet, used in our experiments, was composed of: 18 gm. vitamin-free casein; 65 gm. corn starch; 5 gm. hydrogenated vegetable oil (Crisco); 2 gm. cod liver oil; 6 gm. dried brewers yeast; and 4 gm. salt mixture #1. Control animals were kept on Ralston Purina chow.

For the blood plasma ascorbic acid determination, the technic of Farmer and Abt (5–7) was employed. Blood from the tail of a rat or a mouse was mixed with potassium oxalate, centrifuged, deproteinized with fresh 5 per cent metaphosphoric acid solution, mixed, centrifuged again, and titrated with 2,6-dichlorophenolindophenol. For the tissue ascorbic acid determination, the various fresh organs were weighed, extracted with cold 4 per cent trichloroacetic acid, ground in a Ten Broeck tissue grinder, diluted, centrifuged, and filtered. Ascorbic acid was determined by the method of Roe and Kuether (21) with dinitrophenylhydrazine and checked by the method of Ponting (19). The animals receiving D-gluco-ascorbic acid were fasted for 24–36 hours before ascorbic acid determinations.

For the citrovorum factor determination livers were removed, slices prepared with a Stadie-Riggs microtome and placed in 20-ml. beakers in regular sequence. Krebs-Ringer phosphate solution of pH 7.2 was added. The beakers were

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agitated for 2 hours in a 37° C. water bath. After heating at 100° for 7 minutes, the contents of each beaker were ground in a homogenizer, diluted to 25 cc. with pH 6.4–6.5 buffer, steamed for 2 minutes at 90°, filtered and assayed by the method of Sauberlich and Baumann (22), with *Leuconostoccitrovorum*. Urine was collected under toluene during successive 48-hour periods and assayed for citrovorum factor according to Sauberlich and Baumann's method.

D-Glucoascorbic acid was used in a pure powdered form and added to the Sherman-LaMer scorbutogenic ration.[1]

RESULTS

Toxicity bioassays.—Four groups of 30 male British white-brown rats, approximately 6 months of age, were used. Group I received Purina chow; Group II, the Sherman-LaMer scorbutogenic diet; Group III, the Sherman-LaMer diet to which 1 per cent of D-glucoascorbic acid was added; and Group IV, the Sherman-LaMer diet to which 2 per cent of D-glucoascorbic acid was added. The rats were fed ad libitum. They were weighed before and every 20 days during the 80-day experiment. Table 1 summarizes the results.

TABLE 1

TOXICITY BIOASSAYS
(30 male rats per group)

Group	Av. weight, gm.			Net gain (gm.)
	Initial	40 days	80 days	80 days
I	105	177	218	113
II	107	176	216	104
III	111	178	211	100
IV	108	175	204	96

The average weight gain for Group II, fed the Sherman-LaMer diet for 80 days, was 3.8 per cent less than in the control Group I on Purina chow. The average weight gains in Group III on the Sherman-LaMer diet with 1 per cent of D-glucoascorbic acid and in Group IV with 2 per cent of the antimetabolite were, respectively, 11.3 per cent and 15.2 per cent less than in the control group. This difference between the control and treated groups was expected, but it was not large enough to indicate a markedly toxic effect of the ascorbic acid analog in these amounts. Moreover, there were no apparent manifestations of toxicity such as diarrhea or sluggishness, and no pathologic changes in organs or tissues were observed at autopsy when the experiment was terminated. The addition of 5 per cent or 10 per cent of D-glucoascorbic acid to the Sherman-LaMer diet produced a definite toxic effect with considerable loss of weight, diarrhea, and alopecia. The mortality rate in a group of 30 rats kept on the Sherman-LaMer diet with 10 per cent of D-glucoascorbic acid for 80 days was 40 per cent and 20 per cent in a comparable group given 5 per cent D-glucoascorbic acid. In view of the toxicity of these higher doses, we limited our experiments on cancerous animals to smaller doses (1–2 per cent) which we considered, for all practical purposes, nontoxic.

The ascorbic acid concentration of blood plasma.—One hundred male rats, averaging 8–9 months in age, were divided into four groups as in the previous experiment. The blood plasma ascorbic acid content was estimated on five rats of each group before they were placed on the various regimens, and blood was taken from ten different rats of each group during the 80-day diet period. Before taking blood, the animals were placed on a 24–36-hour fast, since the presence of D-glucoascorbic acid in blood interfered with the estimation of the ascorbic acid content.

The figures of Table 2 show that the Sherman-LaMer scorbutogenic diet slightly reduces the ascorbic acid concentration in blood plasma: from an average of 0.76 mg/100 cc for the rats on Purina chow to 0.61 mg/100 cc of blood plasma for the rats kept on Sherman-LaMer diet. D-Glucoascorbic acid added to the Sherman-LaMer diet at a level of 1 or 2 per cent brought the ascorbic acid concentration of blood plasma close to zero in 8–10 days.

Similar results were obtained with female rats. The drop in the ascorbic acid values in female rats, kept on the Sherman-LaMer diet was much greater than the one which was observed in males: from an average of 0.44 mg/100 cc for the control group to 0.26 mg/100 cc for the group kept on the Sherman-LaMer diet. In general, the plasma ascorbic acid values for females were lower than for males. The effect of D-glucoascorbic acid on the ascorbic acid concentration of blood plasma in females was the same as in the case of males, since the addition of 1 or 2 per cent to the Sherman-LaMer diet brought the level close to zero.

A single parental injection of 20–30 mg. of glucoascorbate caused a slight decrease in the ascorbic acid concentration of blood plasma, bringing it down temporarily to 0.2 mg/100 cc. This was followed by a marked increase in ascorbic acid blood concentration above the normal level, in some instances as high as 0.95 ng/100 cc.

The ascorbic acid levels in spleen, liver, and adrenal.—Sixty male rats, averaging 10 months in age, were divided into four groups as in the previous experiments. Five rats of each group were killed after 10 days, while the remaining ten rats of each group were killed after 30 days. Their livers, spleens, and adrenals were dried *in vacuo* at low temperature and analyzed for their ascorbic acid content. Table 3 summarizes the results.

[1] We wish to thank Dr. Phillip P. Gray and Dr. Harold E. Smith of the Wallerstein Co., New York, for generous gifts of D-glucoascorbic acid.

There was only a slight decrease in the ascorbic acid concentration in liver, spleen, and adrenal from the rats on the Sherman-LaMer diet. The decrease was much more pronounced in the animals kept on the Sherman-LaMer diet with 1 or 2 per cent of D-glucoascorbic acid. Although after 30 days the ascorbic acid concentration in these organs remained higher than that of blood plasma, the ascorbic acid levels for the rats given 1 per cent of D-glucoascorbic acid for 30 days were reduced in the liver to one-thirteenth, in the spleen to about one-thirtieth, and in the adrenals to about one-fifth of those of the analogous tissues of the control animals. This drop was even more evident on a diet with 2 per cent of the antimetabolite.

The antitumor activity of D-*glucoascorbic acid.*—A standard test for activity against tumors was used for D-glucoascorbic acid. This test consists of intraperitoneal injections of the substance under investigation for 7 consecutive days, twice a day, starting 24 hours after transplantation. Mouse Sarcoma 180, Crocker rat carcinoma and mouse adenocarcinoma E 0771 were used. The animals, kept on a regular Purina chow diet, were given intraperitoneal injections of D-glucoascorbic acid, 125 mg/kg of body weight, twice a day for 7 days. The transplants of the tumor fragments were made subcutaneously in the lateral thoracic wall 24 hours before the tests were started. For each test, ten animals were used; five were given D-glucoascorbic acid, while five served as controls. After the test was terminated, the animals were killed, the weights of the tumors recorded, and the ascorbic acid concentrations in the tumorous tissues determined. Table 4 summarizes the results of this test in animals on a diet containing ascorbic acid.

These tests indicate no antitumor activity of D-glucoascorbic acid toward Sarcoma 180 and only

TABLE 4

TEST FOR ANTITUMOR ACTIVITY OF D-GLUCOASCORBIC ACID

(Male animals on Purina chow diet, 5 per group. Daily dose of D-glucoascorbic acid, 250 mg/kg wt for 7 days. Total dose, 1.75 gm/kg wt.)

	Mouse sarcoma 180	Crocker rat carcinoma	Mouse adenocarcinoma E 0771
	Ascorbic acid concentration, blood plasma		
Control:			
before	0.34±0.33	0.82±0.02	0.28±0.07
after	0.35±0.06	0.79±0.09	0.31±0.06
Treated:			
before	0.36±0.05	0.79±0.04	0.32±0.08
after	0.18±0.02	0.54±0.01	0.17±0.02
		Tumor weight, mg.*	
Control	55	98	28
Treated	49	71	21

* Wet wt. 8 days after transplantation.

TABLE 2

BLOOD PLASMA ASCORBIC ACID CONCENTRATION

25 male rats/group

(Av. ascorbic acid concentration in mg/100 cc)

Days	Group I Purina chow control	Group II Sherman-LaMer	Group III Sherman-LaMer plus 1 per cent of glucoasc. acid	Group IV Sherman-LaMer plus 2 per cent of glucoasc. acid
0	0.77±0.042	0.75±0.34	0.78 ±0.041	0.75 ±0.033
5		0.66±0.004	0.33 ±0.002	0.01 ±0.002
15	0.78±0.048	0.63±0.007	0.03 ±0.004	0.013 ±0.003
20		0.61±0.005	0.01 ±0.001	0.008 ±0.001
40	0.76±0.05	0.62±0.01	0.008±0.004	0.006 ±0.002
60		0.56±0.025	0.012±0.001	0.005 ±0.0015
80	0.73±0.042	0.58±0.03	0.009±0.002	0.0058±0.001

TABLE 3

THE ASCORBIC ACID LEVELS OF LIVER, SPLEEN, AND ADRENAL

Male rats

(Ascorbic acid concentration expressed as mg/gm of dry tissue)

Days	No. rats	Group I Purina chow control	Group II Sherman-LaMer	Group III Sherman-LaMer plus 1 per cent of glucoasc. acid	Group IV Sherman-LaMer plus 2 per cent of glucoasc. acid
Liver:					
10	5	1.67±0.34	1.45±0.21	1.30±0.15	1.22±0.22
30	10	1.69±0.45	1.46±0.05	0.11±0.02	0.01±0.005
Spleen:					
10	5	3.12±0.42	2.88±0.32	1.98±0.11	1.66±0.21
30	10	3.05±0.33	2.78±0.02	0.09±0.02	0.02±0.015
Adrenal:					
10	5	17.58±1.12	15.66±0.54	13.21±0.95	11.89±0.98
30	10	18.33±1.12	16.23±1.66	3.55±0.54	0.12±0.11

questionable activity toward Crocker rat carcinoma and mouse adenocarcinoma E 0771. There was only a moderate drop in the ascorbic acid values of blood plasma by the end of testing, and the levels of this substance in blood were quite high.

Since it was essential to find out whether a continuous maintenance of low levels of ascorbic acid in blood plasma and tissue had any effect on tumor growth, additional experiments were conducted with Crocker rat carcinoma, mouse adenocarcinoma E 0771, and guinea pig liposarcoma. Male British white-brown rats, about 10 months of age, average weight 255 gm., were divided into four groups of 30 rats each. The rats were housed separately on screens. Group I, on Purina chow, received 15.0 gm. of the diet/day/rat. Group II, on the Sherman-LaMer ration, Group III, on the Sherman-LaMer diet with 1 per cent of D-glucoascorbic acid, and Group IV, on the same diet but with 2 per cent of D-glucoascorbic acid, each consumed 16.5 gm of diet/day/rat. Thus, the total caloric intake was approximately the same in each of the four groups. Tumor fragments from Crocker carcinoma were transplanted 3 days after the rats were housed. Four weeks after the transplantation the animals were killed, their tumors measured in three dimensions, and the ascorbic acid content of the tumors was determined. The same procedure was applied to C57BL mice, with transplanted adenocarcinoma E 0771. The mice were housed in groups of five: Group I, control, consumed 2.8 gm. of diet/day/mouse, and each of the three treated groups was given 3.1 gm. of the ration/day/mouse. Table 5 summarizes the results.

TABLE 5

THE EFFECT OF D-GLUCOASCORBIC ACID ON CROCKER RAT CARCINOMA AND MOUSE E 0771

(120 male rats, and 120 C57 male mice. Four weeks after transplantation)

	Crocker rat carcinoma		Mouse adenocarcinoma E 0771	
	Av. size (c. cm.)	Av. ascorbic acid conc. (mg/100 gm)	Av. size (c. cm.)	Av. ascorbic acid conc. (mg/100 gm)
Group I, Purina chow	42.5	53.6±1.35	23.2	32.3±2.34
Group II, Sherman-LaMer	40.3	43.4±1.02	20.4	28.4±1.98
Group III, Sherman-LaMer plus 1 per cent glucoasc. acid	20.9	4.98±0.22	11.8	2.67±0.56
Group IV, Sherman-LaMer plus 2 per cent glucoasc. acid	15.4	2.97±0.35	7.7	1.82±0.98

The figures of Table 5 indicate that while the Sherman-LaMer diet had no apparent effect on tumor growth, D-glucoascorbic acid, added to the diet at a 1 or 2 per cent level, exerted considerable inhibitory effect on Crocker rat carcinoma and on mouse adenocarcinoma E 0771. The tumor tissues of rats and mice kept on the Sherman-LaMer ration with 1 or 2 per cent of D-glucoascorbic acid added were considerably, but not completely, depleted of ascorbic acid.

The next series of experiments comprised three groups of six guinea pigs each of the family 2.[2] All animals were fed the Sherman-LaMer diet. Group I received no additional treatment; Group II received daily injections of 40 mg. of sodium ascorbate; and Group III was given daily injections of 40 mg. of sodium ascorbate and 30 mg. of D-glucoascorbic acid. Twenty-four hours before these experiments were begun, liposarcoma was transplanted to the animals of all three groups. One animal of each group was killed after 2 weeks on experiment. The guinea pigs of Group I were killed on the 30th day with two of them in a moribund state, while the rest of the animals were kept until the end of the 5th week of experimentation.

Chart 1 shows that there was an insignificant difference in the average weight of the tumors in Groups II and III. The tumors of guinea pigs receiving ascorbic acid (Group II) weighed an average of 26.5 gm., while the average weight of tumors of Group III receiving both ascorbic acid and D-glucoascorbic acid was 24.2 gm. The growth of liposarcoma in guinea pigs on the Sherman-LaMer diet (Group I) was very slow, and at the end of 30 days the average tumor weight was only 7.4 gm. The ascorbic acid values of the tumor tissue were as follows: Group I, 0.05 ± 0.02 mg/100 gm tissue; Group II, 23.25 ± 1.55 mg/100 gm tissue; and Group III, 19.12 ± 3.24 mg/100 gm tissue. Under the above described conditions it appears that D-glucoascorbic acid did not exert an appreciable antagonistic activity against injected ascorbic acid and produced no inhibitory effect on tumor growth. A vitamin C-free diet, however, retarded considerably the growth of liposarcoma and

[2] We would like to express our gratitude to Dr. W. E. Heston of the National Cancer Institute for the generous supply of guinea pigs of family 2 and for the transplantable tumor, liposarcoma.

brought the ascorbic acid concentration of the tumor tissue close to zero. This observation confirms the findings of Robertson *et al.* (20) concerning the effect of a scorbutogenic diet on the growth of fibrosarcoma in guinea pigs.

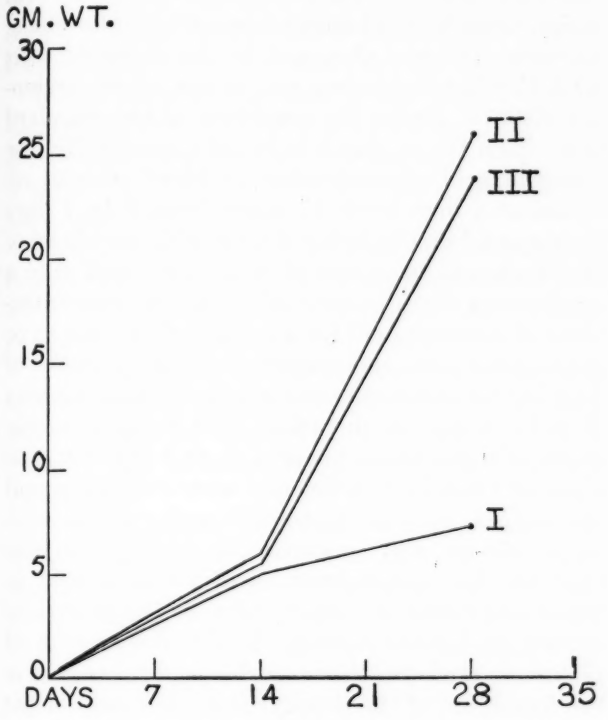

CHART 1.—Growth of liposarcoma of guinea pig, Family 2. Each line gives the average tumor weight for five animals. *I:* Sherman-LaMer diet. *II:* Sherman-LaMer diet and daily injections of 40 mg. of sodium ascorbate. *III:* Sherman-LaMer diet and daily injections of 40 mg. of sodium ascorbate and 30 mg. of D-glucoascorbic acid.

The effect of D-*glucoascorbic acid on the conversion of folic acid to citrovorum factor.*—It was suggested that a reductive step may be involved in the enzymatic synthesis of citrovorum factor (27). Nichol and Welch (16, 17), Broquist *et al.* (2), Hill and Scott (12), and Welch *et al.* (28) found that ascorbic acid, a powerful reducing agent, enhanced the formation of this factor from folic acid. This section concerns the effect of D-glucoascorbic acid on the conversion of folic acid (PGA) to citrovorum factor (CF). Nichol and Welch (16, 17) reported the augmentation by glucoascorbate of the enzymic conversion of PGA to CF by liver tissue *in vitro*, and Welch *et al.* (28), administering glucoascorbate parenterally to rats, found the analog "to be as active as ascorbic acid" in this respect. Sauberlich and Baumann (22) demonstrated that the urinary excretion of CF by rats is proportional to the amount of PGA ingested.

A group of female British white-brown rats, 7–8 months of age, were raised on a purified diet consisting of: vitamin-free casein, 18; Cerelose 67.5; Celluflour, 2; cystine, 0.2; succinylsulfathiazole, 2; vegetable oil, 4; fortified corn oil, 2; choline chloride, 0.1; salts, 4; thiamine, riboflavin, pyridoxine, nicotinic acid, and calcum pantothenate, each 0.3 mg/100 gm of ration; and containing 50 μg of PGA/100 gm. The animals were also fed this diet during the experiment. Six groups of rats, each containing five animals, were used. The rats of Groups IV, V, and VI were kept on this purified diet supplemented with 1 or 2 per cent of D-glucoascorbic acid for 10 days prior to the experiment. The ascorbic acid concentrations of blood plasma in these rats were brought down close to zero. Urine from each rat was collected under toluene during 48-hour periods and assayed with *Leuconostoc citrovorum*.

The results of these trials are summarized in Table 6.

Several facts emerge from these observations.

1. In confirmation of Nichol and Welch (14), the addition of ascorbic acid to a purified diet considerably enhanced the conversion of PGA to CF. In-

TABLE 6

THE EFFECT OF D-GLUCOASCORBIC ACID ON URINARY EXCRETION OF CITROVORUM FACTOR

(Female rats on a purified diet containing 50 mg/100 gm of folic acid. Av. 48-hour amount of CF for four collections, five rats.)

	Units
Group I: purified diet only	53
Group II: purified diet plus 1.0 mg. of PGA daily	3,700
Group III: purified diet plus 1.0 mg. of PGA and 0.1 mg. of ascorbic acid daily	18,450
Group IV: purified diet with 1 per cent of D-glucoasorbic acid plus 1.0 mg. of PGA daily	1,005
Group V: purified diet with 2 per cent of D-glucoascorbic acid plus 1.0 mg. of PGA daily	697
Group VI: purified diet with 1 per cent of D-glucoascorbic acid plus 1.0 mg. of PGA and 0.1 mg. ascorbic acid daily	17,000

stead of an average amount of 3,700 units of CF (group II) collected during the 48-hour-period, 18,450 units were present in the urine collected during the same period of time when ascorbic acid was added to the diet (Group III), or the amount of CF was augmented fivefold.

2. When a purified diet containing 1 or 2 per cent of D-glucoascorbic acid was used and the ascorbic acid level was brought close to zero, not only was there no increase in the conversion of

PGA to CF but the amount of CF was much smaller than in the control group of rats receiving no ascorbic acid. Instead of the 3,700 units collected from rats given 1.0 mg of PGA (Group II), the urine collected from rats on a diet containing 1 per cent of the analog and receiving PGA contained 1,005 units (Group IV), or less than one-third as much. Thus, apparently, the low concentration of ascorbic acid in blood induced by continuous feeding of the analog impedes, at least partially, this conversion. A single parenteral injection of the analog does not affect to any great degree the ascorbic acid concentration of blood in rats and, according to Welch *et al.* (28), apparently does not impede the conversion of FA.

3. The enhancing effect of dietary ascorbic acid on the conversion of PGA to CF was not abated by presence of the analog in the diet. This is apparent from the results obtained with group VI, when the rats were kept on a diet containing 1 per cent of D-glucoascorbic acid and received a daily dose of 1.0 mg. PGA and 0.1 mg. of ascorbic acid. The average amount of CF collected during a 48-hour period was 17,000 units or about the same as that when ascorbic acid was given alone, without its analog.

DISCUSSION

Woolley (30) tends to admit that the competitive action of D-glucoascorbic acid is not clearly manifested and that no inhibition index can be established in regard to the ascorbic acid-glucoascorbic acid antagonistic activity. He still adheres to his opinion, expressed in 1943, that D-glucoascorbic acid induces a scurvy-like condition in rats, mice, and guinea pigs, although this assumption has been contested by other investigators (Gould [11], Gorlin [10], Schafer [23]).

Our observations have not disclosed any antagonistic activity of D-glucoascorbic acid to dietary vitamin C but have revealed that when this analog was administered in relatively small doses (1 or 2 per cent added to a purified diet) it decreased the ascorbic acid concentration in blood plasma, liver, spleen, and adrenals in rats and mice to values close to zero without inducing any of the toxic manifestations reported by Woolley and other investigators working with larger doses of this substance.

Thus, apparently a low concentration of ascorbic acid in blood and tissues in rats and mice does not necessarily induce any signs of vitamin C deficiency in these animals. They remain in good health after many months of this diet. It appears doubtful that a true scurvy could be induced in rats or mice by administration of the analog.

On the other hand, D-glucoascorbic acid appears to interfere with the biosynthesis of ascorbic acid, possibly by a suppressive action upon the ascorbic acid-forming tissue. The nature of this action has not yet been determined.

Our standard test with D-glucoascorbic acid on Sarcoma 180 disclosed no antitumor activity. An insignificantly small antitumor activity of D-glucoascorbic acid was observed in the standard tests with Crocker carcinoma and mouse adenocarcinoma E 0771. Under the condition of the standard test, when the analog is injected parenterally, the ascorbic acid concentration in blood plasma remains at a high level. It takes from 5 to 7 days on a special diet to bring the ascorbic acid level of blood plasma to values close to zero, and only a continuous maintenance of such low concentrations of ascorbic acid for a period of two weeks or more produces retardation in the growth of Crocker rat carcinoma and mouse adenocarcinoma E 0771. Since, in the trials with small nontoxic doses of D-glucoascorbic acid (1 or 2 per cent), no signs of vitamin C deficiency were evidenced and the animals were asymptomatic as far as the scurvy syndrome was concerned, one may conclude that the low concentrations of ascorbic acid in blood and tissue are unfavorable to the growth of certain malignant tumors. If the observation of Goldstein *et al.* (9), that ascorbic acid takes part in the formation of the desoxyribonucleic acid of the cell nucleus is confirmed, we might find a proper interpretation of the phenomenon described by us.

In man, a scorbutogenic diet or a diet low in vitamin C abates the conversion of folic acid to citrovorum factor, according to Broquist *et al.* (2), Gabuzda *et al.* (8), and Welch *et al.* (28). It may be added that, according to Crandon *et al.* (4), the first symptoms of scurvy syndrome appear in man only after more than 4 months on a vitamin C-free diet. They observed that the plasma ascorbic acid concentration dropped to and remained at zero after 41 days of the experimental vitamin C-free diet, even though the abnormal clinical signs of ascorbic acid deficiency, such as hyperkeratotic papules, did not appear before 132 days. Burrill (3) and Stamm *et al.* (27), on the basis of their extensive investigations of gingivitis, concluded that there is no justification for ascribing this condition to vitamin C deficiency alone, and that serum levels near zero are not indicative of scurvy. The observation of Miller *et al.* (15), who kept their patients on a vitamin C-free diet for a period from 4 to 6 months without any apparent signs of scurvy, confirm the previous findings of other investigators and suggest that this regime might be safely used for lowering the CF conversion for a period up to 6 months.

SUMMARY

When D-glucoascorbic acid was added to a Sherman-LaMer scorbutogenic diet at a level of 1 or 2 per cent, no apparent toxic effects such as diarrhea, alopecia, or hemorrhagia in rats and mice was produced, but on this diet the ascorbic acid concentrations of the blood plasma and certain tissues were lowered to nearly zero in 20 days.

Intraperitoneal injection of 250 mg/kg/day of D-glucoascorbic acid for 7 days did not retard the growth of mouse Sarcoma 180 and only slightly inhibited the Crocker rat carcinoma and mouse adenocarcinoma E 0771.

Continuous feeding of a Sherman-LaMer ration containing 1 or 2 per cent of D-glucoascorbic acid for 4 weeks considerably retarded the Crocker carcinoma and adenocarcinoma E 0771.

D-Glucoascorbic acid did not affect the growth of liposarcoma in guinea pigs receiving an adequate supply of vitamin C, although the tumor growth was retarded considerably in scorbutic guinea pigs.

In the absence of dietary ascorbic acid, the urinary excretion of the citrovorum factor in rats was decreased by a diet containing 1 or 2 per cent of the analog, but the antagonist did not significantly affect CF excretion in the presence of dietary ascorbic acid.

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A Cytological Study of Colchicine-induced Nodal "Tumors" in Tradescantia

L. Joan McGowan and Charles J. Bishop

(*Biological Laboratories, Acadia University, Wolfville, Nova Scotia*)

Following the treatment of cuttings of *Tradescantia paludosa* with colchicine for the production of polyploidy, abnormal tumor-like swellings were found to develop at most of the nodes. Many of these later became black and underwent degenerative changes, but from some of them young shoots regenerated which produced normal plants. The favorable nature of this material for cytological analysis presented an opportunity of making a study of the development of this tumor-like tissue and the cytological changes connected with its degeneration.

Abnormal morphological development has frequently been found to follow the use of colchicine. Havas (16) observed deformation of wheat seedlings after colchicine treatment, with the formation of bulbous shoots containing "monstrous hyperchromatic nuclei." Dustin *et al.* (12) found that such abnormal nuclei had many nucleoli and were lobed and also that the "tumor" cells eventually tended to collapse and disintegrate.

Similar tumor-like structures at both shoot and root apices have been reported by Nebel and Ruttle (29), Levan (23), Shinamura (30), Emsweller and Brierly (14), Hawkes (18), Levine (27), and Levan and Lotfy (25) following colchicine treatment. These were generally found to develop as a result of an increase in cellular size rather than by cell proliferation, though Hawkes (18) considered that enlargement was due to a disturbance of the polarity of cell elongation.

From this it may be concluded that colchicine treatment of plant tissue can frequently result in abnormal overgrowths which somewhat resemble tumors of plants or animals. This paper is an attempt to assess the significance of the cytological changes which occur in their development and to determine the cytological causes of the tissue degeneration.

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MATERIALS AND METHODS

This study was carried out on No. 1 Acadia clonal line of *Tradescantia paludosa*, Anders. & Woodson, which has a diploid chromosome number of 12. For treatment, two-node cuttings were made, the inflorescences removed, and the cuttings set in moist sand. Three days later, they were immersed completely in a solution of 0.2 per cent colchicine in distilled water. The treatment extended over 48 hours, after which interval the cuttings were rinsed and again planted in moist sand. At selected intervals ranging from 24 hours to 48 days nodes were removed and fixed in Craf IV. Serial sections were cut at a thickness of 20 μ and stained with Heidenhain's hematoxylin.

RESULTS

Macroscopically, no effect from the colchicine treatment was noted until about 1 week after the cuttings were removed from the solution, when some swelling of one side of the nodal portion was usually observed. By about 2 weeks this had enlarged to a rounded compact structure, shorter and of greater diameter than the corresponding control shoots. This enlargement continued for about 1 month, when an average diameter of about 5 mm. was reached. After this period, the nodal growth either atrophied or a shoot, similar in appearance to the controls, grew out from the middle of the "tumor" tissue. Some of these shoots were permitted to grow and flower, and the chromosome numbers of developing pollen grains checked by making anther smears. While two of six inflorescences thus examined had twelve chromosomes (tetraploid) at pollen grain division, the other four were diploid.

At the end of 1 week the shoots on treated cuttings were noticeably larger than those of the controls. Microscopic examination showed that this was owing completely to an increase in cell size as a

result of polyploidization. There were the same number of leaf layers in both shoots, but in the control material each layer was composed of small compact cells, differentiated into epidermal and parenchymatous tissues with developing vascular bundles (Fig. 1), while shoots of the treated nodes

Chart 1.—Camera-lucida diagram of a longitudinal section through a typical colchicine-induced "tumor" in Tradescantia. ×20.

contained a relatively small number of enlarged, irregularly shaped cells (Fig. 2). Such cells were usually packed together in an undifferentiated mass, with little or no development of vascular elements.

While the cell size of the controls varied somewhat, depending upon the region of the shoot in which they were located, the nuclei were all of the same size. The nuclei in cells of the treated shoots were often much larger than those of the controls, were made up usually of several lobes, and contained many nucleoli (Fig. 3). There was considerable variation in nuclear size, and chromosome numbers ranging from 12 (2n) to almost 200 (32n) were counted in the same node. There was little localization of cells with any one chromosome complement. All divisions observed were apparently quite normal, even in the highly polyploid cells (Fig. 4). The spindles appeared to be bipolar, and no multipolar spindles were seen.

A typical "tumor" at this stage consisted of about five leaf layers surrounding a central dome of meristem (Charts 1 and 2). The outermost layer consisted of leaf mesophyll with a distinct epidermis. There were no nuclear divisions in this layer, but, since the nuclei and cells of both tissues were larger than those of the main shoot and many nuclei were lobed, they were considered to be at least tetraploid. The second layer was made up completely of a relatively small number of large, irregularly shaped cells with huge lobed nuclei, indicative of a high degree of polyploidy. The three inner layers and the meristem are shown in part in Figure 2. All but the small central region were composed of similarly large and irregular cells with

Chart 2.—Camera-lucida diagram of a longitudinal section through a normal shoot of Tradescantia. ×20.

lobed nuclei. From actual chromosome counts and from approximations based on nuclear size it was concluded that the chromosome numbers ranged from 48 (8n) to 192 (32n), most of the cells being 8n or 16n. The central meristematic zone, however, consisted of a few small, rounded cells with nuclei which appeared diploid, although no divisions could be counted. Between this region and

the stem of the cutting lay more mixoploid tissue, comprised of a small number (perhaps 5 per cent) of 2n cells, mixed with 4n to 16n cells. Although no mature tracheids were noted, groups of elongated cells in the basal region indicated some differentiation of that type.

Such a structure was characteristic of most of the nodal growths fixed at the later stages. However, by 4–5 weeks after treatment, some of the cells with very highly polyploid nuclei appeared to degenerate, although those with lower chomosome complements (2n, 4n, 8n) were still dividing. At these later stages, the central meristematic region was usually composed exclusively of 2n and/or 4n cells.

The oldest shoot examined microscopically was fixed 48 days after colchicine treatment. This shoot consisted of outer layers of large, collapsed, and apparently degenerating parenchymatous cells with very large lobulated nuclei. Inside this region, a new shoot was developing. This shoot was made up almost entirely of diploid cells, with a few 4n cells intermixed. Between the diploid tissue and the stem of the original cutting lay an area of mixoploid tissue, with cells 2n to 32n.

The colchicine treatment apparently induced a succession of c-mitoses persisting for several days, for active spindle formation was not definitely observed until 13 days after the conclusion of the treatment, and the cells became progressively of a higher polyploid condition until about that time. In this manner, tissues of a mixoploid nature were produced. However, some of the cells showed no evidence of polyploidy, and these lay either in the central meristem or in the tissue between this and the stem of the original cutting. While the higher polyploid cells with their extreme size and lobulate nuclei appeared to be no longer capable of normal cell division and degenerated, the cells of low chromosome number were able to divide normally and formed a new shoot of either diploid or tetraploid tissue. Thus, the outcome of colchicine treatment appeared to be degeneration of the highly polyploid cells and regeneration from any diploid or tetraploid tissue remaining.

DISCUSSION

It has been noted that the increase in shoot size causing a tumor-like growth was due to the enlargement of cells, rather than to a cellular proliferation. In the leaf primordia the colchicine action persisted until, in some cases, a 32n degree of polyploidy was reached. Later, spindle formation resumed, and normal cell division took place, at least in the cells of lower polyploidy. These observations agree with those of Levan (22, 23), who produced "tumors" on both roots and shoot apices as a result of increases in the volume of the meristematic cells and without the formation of new cells. Similar effects have been noted by Shinamura (30), Dermen and Bain (9), and Levan and Lotfy (25). Tumor formation of this type, then, is due to the inhibition of cell division by the action of colchicine, and not to an abnormal stimulation of growth.

In this respect, colchicine-induced "tumors" are not comparable to plant overgrowths produced by other agents, such as growth-promoting substances. These agents usually produce a callous tumor-like mass resulting from cell proliferation. Carcinogenic substances and the crown gall organism induce overgrowths of the same type. Some degree of polyploidy has been noted in such plant tumors (Greenleaf [15], Dermen [8], and Levan and Lotfy [25]), although it was not considered to be produced by inhibition of the mitotic spindle, but rather due to the increase by cell division of a condition already existing in the plant tissue. Huskins (19) and Huskins and Steinitz (20) have shown that endopolyploidy may occur under natural conditions and that growth substances merely stimulate the cells to divide and thus show up the natural polyploid condition. Coleman (7) expressed this view in his studies on *Vicia faba*, and it is also supported by the cytochemical work of Swift (31).

One of the most striking features of the colchicine "tumors" was the large lobed nuclei found in most of the cells (Fig. 3). The method of their development, aside from the increased chromosome complement, is unknown. Formation by multipolar spindles is considered unlikely, for these were never observed, and such divisions usually result in formation of several small cells (Levan and Lotfy [25]). It seemed more likely that the lobed condition was merely a means of increasing the nuclear surface in proportion to its large volume. Each of the lobes was connected at a central point, and each contained at least one of the prominent nucleoli noted in all Tradescantia cells.

Prophase stages of lobed nuclei were occasionally observed, and, as the chromatin threads became distinguishable, the lobed nature was apparently lost. However, since lobulation of nuclei was not so marked in areas where divisions were most common, i.e., in and near the meristem, and since divisions of such nuclei appeared to be infrequent, it was concluded that these large and deeply lobed nuclei could undergo little further division.

Eigsti (13) and Shinamura (30) have reported both compound lobed nuclei and multinucleate cells following colchicine treatments, and Hawkes

(18) noted nuclei that were "fragmented," apparently owing to irregularities in the reorganization of the restitution nucleus. However, no true multinucleate cells were observed in the Tradescantia material of the present research.

Polyploid cells with such lobed nuclei have been observed in animal tumors (Levine [26]), and seem to be a frequent component of advanced malignant growths. The exact relation between polyploidy and cancer is still in doubt, even though Boveri (5) early considered that cancer cells with an unlimited growing power developed only as a result of abnormal chromatin content due to chromosome multiplication and the loss of "retarding" chromosomes from the cell.

In the present research, the degeneration of the highly polyploid cells with lobed nuclei was noted as the final stage of development of the Tradescantia "tumors." Such degeneration of colchicine-induced polyploid cells was observed by Havas (16) and Dustin *et al.* (12). Biesele *et al.* (3) proposed that aneuploidy and gene unbalance may be associated with the development of oversized nuclei, and these factors may result in the death of the cells, a condition characteristic of many animal tumors. Levine (26) suggested that in animal tumors the products of this degeneration may stimulate other cells to divide.

As a practical application of the degenerative action of colchicine, investigations have been made of the use of colchicine as a therapeutic agent in cancer treatment (see Levine [28]). In plants, this drug has been shown to inhibit the growth of developing galls and to destroy mature ones (Havas [17], Brown [6], and Dermen and Brown [10, 11]) by greatly increasing the cell volumes until the growths eventually dried up.

Although colchicine-induced "tumors" do not resemble closely other plant tumors, they do share certain features with animal malignancies. Koller (21) has outlined some of the bases for this comparison including polyploid, multinucleate, and giant cells, multipolar spindles, and increased division rates due to a change in nucleic acid synthesis. Thomas (32) added to these an increase in chromosome size and the enlargement of the nucleolus.

While the colchicine-induced "tumors" studied in this work should not be considered as analogous to animal tumors, they did show some of these cytological similarities. These included particularly the increased nuclear size and its irregularity, and the increased chromatin content as a result of the polyploidization, both of which have been found in certain types of animal tumors (Levine [26], Biesele and Poyner [2], Biesele [1], and Levan and Hauschka [24]). The possible significance of this polyploidy in cancer therapy has been pointed out previously (Bishop [4]).

In the early stages of the colchicine-induced "tumors" there was apparently very active production of polyploidy by chromosome division, although it appears that the chromosomes later ceased dividing. No reversion to a lower chromosome number was observed, and it is not thought that such would take place unless through the activity of multipolar spindles, none of which were seen. Thus, although growth activity is limited, it does resemble a true tumor in that it is not reversible to the normal type.

It was also apparent that, once the cells reached the abnormal lobulate condition and ceased division, they began to degenerate and eventually died. In this respect they resemble cells in the advanced stages of some types of animal malignancies. On the other hand, most plant tumors do not show any such degeneration.

From this comparison it would seem possible that the developmental process of certain animal tumors may follow a pattern somewhat similar to that found in these colchicine-induced plant "tumors." Normal cells in each case become changed through some abnormality in cell division causing an increase in chromosome number, either as a result of the lack of chromosome separation or by endomitotic chromosome duplication. Finally, complex polyploid cells with abnormal lobulated nuclei are formed, which in their extreme development are unable to function normally and eventually die. This disintegration, as suggested by Levine (26), may liberate some chemical stimulus for the division of surrounding cells. Alternatively, it might cause in them the initial mitotic upset leading to the abnormal highly polyploid condition. In extreme cases the cellular breakdown will result in disorganization of tissues and may cause the death of the organism.

SUMMARY

Tumor-like growths were produced at the nodes of cuttings of *Tradescantia paludosa* by complete immersion for 48 hours in a 0.2 per cent aqueous solution of colchicine. Histological and cytological features of the developing "tumors" are presented, and it is concluded that they arose through a series of colchicine-inhibited mitoses in the cells of the shoot and leaf primordia. These resulted in a mixoploid tissue with cellular chromosome complements ranging from 2n (12) to at least 32n (192). While the lower chromosome number cells were capable of division, and in some cases gave rise to diploid or tetraploid branches, the highly poly-

ploid tissue degenerated, causing the "tumors" to atrophy. Cytological similarities to animal tumors are pointed out, and it is suggested that the process of development and degeneration in the two cases may be cytologically similar.

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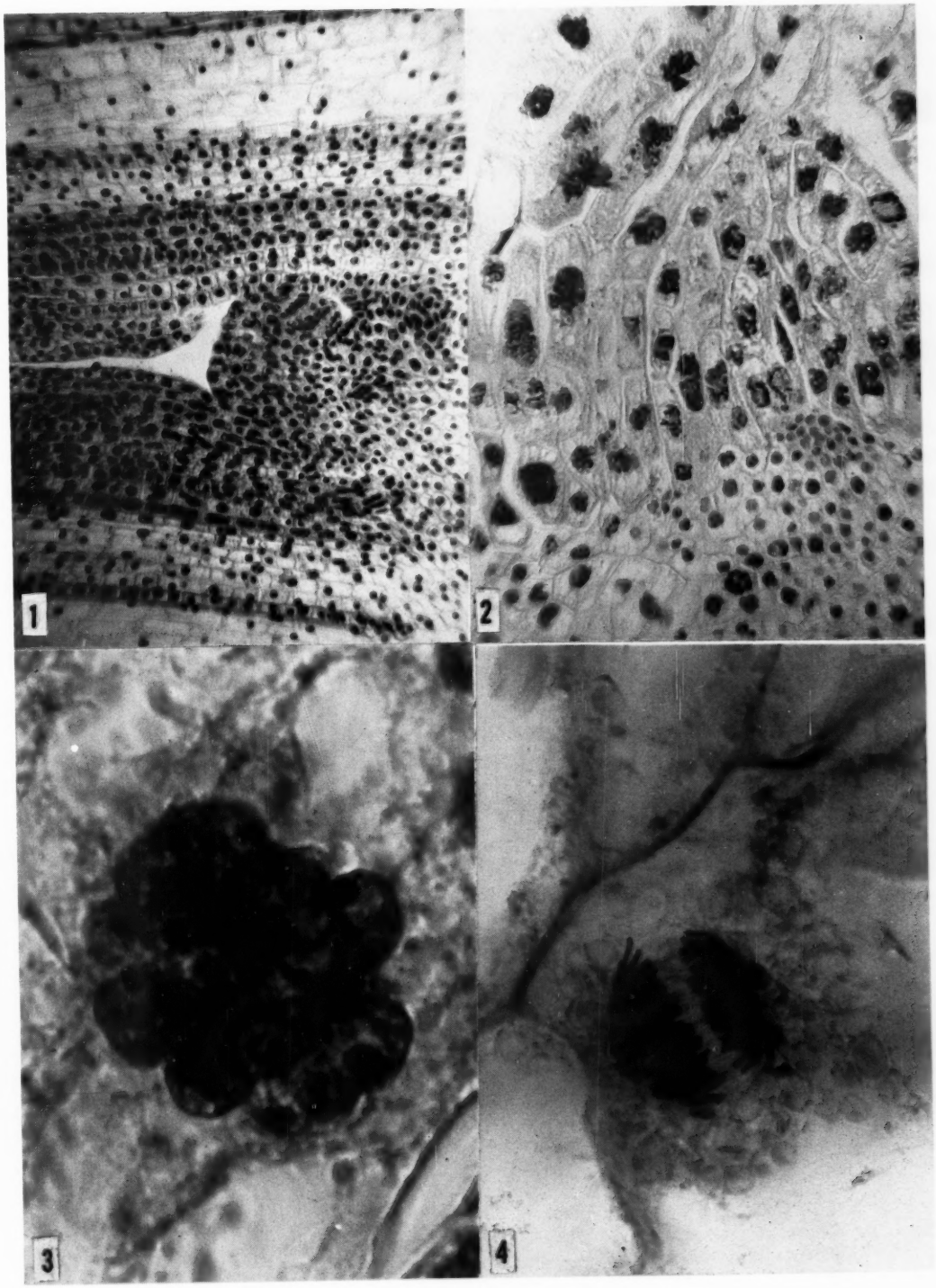
Fig. 1.—Photomicrograph of a section through an untreated Tradescantia shoot at 19 days. ×100.

Fig. 2.—Photomicrograph of a section through a colchicine-treated shoot of Tradescantia, 19 days after treatment. ×100.

Fig. 3.—Highly polyploid cell with abnormal lobulate nucleus. ×900.

Fig. 4.—Anaphase stage of a polyploid nucleus containing 192 (32n) chromosomes. ×480.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Morphologic and Biologic Changes in a Mouse Ascites Tumor Following Induced Infection with Certain Viruses*

IRENA KOPROWSKA AND HILARY KOPROWSKI

(Papanicolaou's Cytology Laboratory, Department of Anatomy, Cornell University Medical College, New York City, and Viral and Rickettsial Research, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N.Y.)

It has been previously reported in a study on the effect of neurotropic viruses upon transplantable mouse tumors (8) that each of these viruses had its own tumor spectrum, i.e., it destroyed or inhibited certain mouse tumors, multiplied without damage in others, and failed to multiply in some (8). On the basis of these animal experiments, particularly in relation to the effect of Bunyamwera, West Nile, and Ilheus encephalitis viruses upon malignant growth, preliminary work has been done elsewhere on the treatment of human patients with these three viruses (16, 17).

Evaluation of the oncolytic activity of a viral agent upon a solid transplantable tumor was sometimes a difficult problem for the following reasons: The neoplastic tissue was not homogeneous, because central necrosis was present, and therefore the accessibility of cells to viral infection differed in various portions of the tumor. Since the results were based upon histologic examination and/or transplantation of only small fragments of the malignant tissue, it was not possible to judge the effect of the virus upon the tumor *in toto*. In addition, it was impossible to observe and follow closely the actual process of "invasion" of the tissue by a given virus, since daily biopsies of the same individual were not feasible, and, therefore, this mechanism of the viral action could not be well understood.

Many of the above difficulties inherent in working with a solid tumor mass could have been overcome had it been possible to employ *in vivo* suspensions of malignant cells similar to those observable in tissue culture technics *in vitro*. Such a type of malignant neoplasm was found in the so-called Ehrlich ascites tumor, the history of which has been described in detail by Klein (5).

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At the time the present work was initiated, there were few references to the biologic aspects of the use of ascites tumors (3, 4, 9). The present authors were the first to report on the use of the ascites tumor as a tool in the general study of the tumor-virus system (6). This paper represents a more detailed report on the effect of several viruses upon this ascites tumor, with particular emphasis upon the oncolytic action of the Bunyamwera virus.[1]

MATERIALS AND METHODS

Viruses.—The following viruses were tested: Bunyamwera, eastern and western equine encephalomyelitis, GDVII strain of mouse encephalomyelitis, Ilheus, influenza A (neurotropic WS strain), Japanese B encephalitis, louping-ill, rabies, Semliki forest, St. Louis encephalitis and West Nile (M956 and Egypt 101 strains).

The viral inocula were mouse brains stored in the form of 40 per cent suspensions in distilled water in a frozen state at −60° to −70° C. Unless otherwise stated, each tumor-bearing mouse was inoculated intra-abdominally with 0.5 ml. of viral suspension. The concentration of infected brain tissue in the inoculum varied as stated in each protocol. The diluant of the mouse brain suspension was physiologic salt solution.

Tumors.—The Ehrlich ascites tumor was used throughout the work. It was at the time unavailable in this country and was sent to us on June 1, 1950 by Dr. Albert Fischer, of the Biological Institute of the Carlsberg Foundation, Copenhagen, Denmark, through the kindness of Dr. Herdis von Magnus.[2] The tumor was maintained in our laboratories in serial passages in the peritoneal cavities of either Swiss albino or Strong A mice. The tumor was usually grown for 6–9 days, and the resulting ascitic fluid, obtained either by tapping the abdominal wall or by aspiration from the peritoneal cavity after the animal had been sacrificed, was used as inoculum for the next series.

Technics.—In the experiments 0.2 ml. of undiluted ascitic fluid, containing not less than 10 million tumor cells (unless otherwise stated), were injected intra-abdominally into groups of Swiss albino, PRI (see below) or Strong A mice. Four days later one-half of the tumor-bearing mice was infected with the

[1] Preliminary results of the study were presented at the 37th Annual Meeting of the American Society for Experimental Pathology in New York City, on April 15, 1952 (6).

[2] At a later date the authors obtained the Ehrlich ascites tumor from Dr. G. Klein, of the Karolinska Institutet, Stockholm, Sweden, to whom they are greatly indebted for his kindness and cooperation.

respective viral agent and another group, used as control, was injected with normal mouse brain suspension. Four to 6 days later, when the virus-inoculated mice showed signs of illness, the abdominal cavity was tapped, and all ascitic fluid obtainable was aspirated. Each separate specimen of the fluid was measured and pooled with those obtained from two or three other animals of the same group. The mice infected with virus and the control mice were observed until they died, or for a period of 60 days. The presence of ascites and/or solid tumor of the peritoneal cavity was noted at the death of the animal.

Bioassay.—Aliquots of each pool of ascitic fluid were injected subcutaneously into mice for bioassay. Five or six mice were used for each sample, and each mouse received 0.2 ml. of undiluted fluid. In addition, in some cases serial tenfold dilutions of the ascitic fluid were made in physiologic salt solution, and aliquots of each dilution were inoculated subcutaneously into mice. Control fluids and fluids infected with virus were assayed at the same time.

The growth of subcutaneous tumors in the inoculated mice was observed frequently, and animals which either failed to develop tumors or developed them and survived were observed for 60 days.

The results of the bioassays are given in terms of ratios of mice that developed tumors after implantation with presumably virus-infected aliquots of the ascitic fluid, and of control mice which received implants of ascitic fluid from mice which were not subjected to the viral treatment. This is referred to in the tables as "Tumor Ratio."

Presence of virus.—The remainder of the pool of ascitic fluid obtained from virus-infected mice was centrifuged in an angle-head International type I centrifuge for 10 minutes at 1,500 r.p.m. Tenfold serial dilutions of the supernatant fluid in physiologic salt solution were injected into Swiss mice in 0.03-ml. amounts by the intracerebral route. In some cases the control fluids were also submitted to the same procedure, but only the undiluted supernatant fluid was injected into mice. This was done to exclude the possibility that the death of some mice might have been caused by the intracerebral growth of tumor rather than by the virus. The injected mice were observed for 21 days, and the number of sick and dead animals was recorded.

Cytologic technic.—Unless otherwise stated, smears for the cytologic study of each pool of the ascitic fluid obtained from the virus-infected mice and the control animals were made in the following manner: One or two drops of the undiluted peritoneal fluid were placed on at least two labeled, clean glass slides and spread uniformly over the surface. Smears were placed immediately without drying into a fixing solution consisting of equal parts of ether and 95 per cent alcohol, in which they remained for at least 24 hours. Fixed smears were stained according to the routine Papanicolaou staining procedure 267 (14).

Differential counts and presentation of results.—At least one hundred, and often two hundred, cells were counted in every smear, and the percentages of malignant cells, histiocytes, polymorphonuclear leukocytes, and lymphocytes were calculated. In addition, in later experiments percentages of mitoses, degenerated cells, and cells with karyorrhexis were recorded. A typical example of a differential count in a control slide is shown in Table 1.

The evaluation of the effect of the infection with virus upon the morphology of the Ehrlich ascites carcinoma cells was made by comparing the differential counts obtained from smears of peritoneal fluid of virus-infected cancer-bearing mice to the corresponding counts in noninfected cancerous mice.

RESULTS

Microscopic appearance of smears from control animals.—The malignant cells generally represented as many as 80 per cent of cells. They were usually uniformly distributed, discrete, fairly well preserved, and easily identified by their large size, large nuclear-cytoplasmic ratio, hyperchromasia, prominent nucleoli, and coarse chromatin distribution. Multinucleation and cytoplasmic vacuolization were occasionally seen in the malignant cells. Mitotic figures were common (1–3 per cent), and multipolar mitosis was often observed. Necrotic cells and karyorrhexis were observed only in rare instances. An occasional malignant cell was noted with pseudopod-like extensions of the cytoplasm beyond the cellular borders.

TABLE 1

TYPICAL DIFFERENTIAL COUNTS FOR A CONTROL SMEAR OF ASCITIC FLUID

PER CENT CA			PER CENT HIST.			PER CENT POLY.			PER CENT LYMPH.			PER CENT MITOSES			PER CENT DEGEN. CELLS			PER CENT KARYORR.		
Slide			Slide			Slide			Slide			Slide			Slide			Slide		
I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
81	79	79	9	9	6	5	9	10	2	2	2	3	1	3	0	0	0	0	0	0

Figure 1 illustrates a typical microscopic appearance of a smear from a noninfected control mouse with Ehrlich carcinoma ascites 9 days after implantation. It will be seen that malignant cells predominated. The remaining cells in the control smears consisted of histiocytes, polymorphonuclear leukocytes, and lymphocytes. The histiocytes exhibited considerable variation in size, although the largest of them only rarely exceeded the size of the malignant cells and were usually smaller than the latter. The larger histiocytes were easily identified by their delicate, "foamy," indistinctly outlined cytoplasm and relatively small nuclear cytoplasmic ratio. The histiocytic nuclei were often eccentric, round, oval, or bean-shaped. The cytoplasm sometimes contained inclusions of phagocytized cellular material. The smaller histiocytes were usually identified by the same criteria, although their distinction from immature polymorphonuclear leukocytes and large lymphocytes was sometimes made in an arbitrary manner. The mesothelial cells, known to constitute an important and sometimes striking component of smears prepared from human serous

effusions regardless of the presence of malignant cells, were not sufficiently distinguishable from histiocytes in this material to be counted as a separate cellular type. Polymorphonuclear leukocytes were usually smaller than histiocytes and were recognized by their characteristic fragmented nuclei. The lymphocytes appeared in the smears as small round cells with relatively large, round, sometimes slightly indented, dark pyknotic nuclei, surrounded by a narrow rim of cytoplasm. The erythrocytes, in general, were numerous in the smears, but were not included in the differential counts.

Infection with viruses which usually failed to multiply in ascites tumor and had no effect on the neoplastic process.—Details of these experiments will be omitted, since early in the work it became apparent that eastern, western equine encephalomyelitis, Semliki forest, and rabies viruses failed to infect the tumor and failed to interfere with the viability of the malignant cells. Table 2 summarizes a few of the experiments, showing that only in two experiments (B and E) were the viruses recovered from the ascitic fluid, and even then they had no effect upon the neoplastic process, as evidenced by the results of bioassay.

Figure 2 illustrates the microscopic appearance of a smear prepared from the ascitic pool of mice infected with the eastern virus. No morphologic differences were seen between these cells and those in the control smears (Fig. 1).

Microscopic examination of smears from Ehrlich ascites tumor infected with western, Semliki forest, and rabies viruses revealed not only lack of any significant decrease of the percentage of cancer cells, but absence of any features of cellular degeneration which might suggest an increased destruction of neoplastic elements. At no time did the number of degenerated cells and cells showing karyorrhexis sufficiently increase to be included in the differential counts. On the contrary, the malignant cells were well preserved and apparently

TABLE 2

EFFECT OF EASTERN, WESTERN EQUINE ENCEPHALOMYELITIS, SEMLIKI FOREST, AND RABIES VIRUSES UPON ASCITES TUMOR IN MICE*

Virus	Exp.	Inoculum: Dilution of virus suspension	Cause of death: Virus infection (M.R.[a])	Cause of death: Ascites tumor (M.R.[b])	Mice tapped (no.)	Amt. of fluid aspirated (ml.)	Cell count: Per cent Ca Slide I	Per cent Ca Slide II	Per cent Hist. Slide I	Per cent Hist. Slide II	Per cent Poly. Slide I	Per cent Poly. Slide II	Per cent Lymph. Slide I	Per cent Lymph. Slide II	Results of transplants: Dil. of fluid	Tumor ratio	Ascitic fluid LD_{50} titer in mice
Eastern equine enc.	A	1:2½	5/5		2	3	68	73	11	11	7	9	14	7	undil.	5/5	<undil.
		None		4/4	1	3	74	98	5	1	3	1	18	0		4/4	
	B	1:2½	1/6	5/5	2	6	73	71	7	5	11	12	9	9	undil.	5/5	$10^{-3.00}$
		None		7/7	2	7	70	72	9	19	1	2	20	7		5/5	
	C	1:20	4/6	2/2	2	5	73	66	14	19	9	8	4	7	10^{-1}	4/4	<undil.
															10^{-2}	4/4	
															10^{-3}	4/4	
															10^{-4}	2/4	
															10^{-5}	1/4	
															10^{-6}	0/4	
		None		6/6	2	6	77	83	6	13	10	1	7	3	10^{-1}	4/4	
															10^{-2}	4/4	
															10^{-3}	4/4	
															10^{-4}	2/4	
															10^{-5}	0/4	
															10^{-6}	0/4	
Western equine enc.	D	1:2½	0/4	4/4	2	8	99	88	0	1	0	0	1	1	undil.	5/5	<undil.
		None		4/4	1	6	76	62	17	13	1	11	6	14		5/5	
	E	1:2½	0/5	5/5	2	6				Not tested					undil.	5/5	$10^{-3.75}$
		None		13/13	2	9										4/5	
	F	1:5	3/6	3/3	2	4	67	66	23	26	9	4	1	4	undil.	8/8	$<10^{-2.00}$
		None		6/6	1	4	91	67	3	4	4	14	2	5		8/8	
Semliki forest	G	1:1000	5/5		2	5	68	82	21	9	7	5	4	4	undil.	5/5	<undil.
		None		4/4	2	10	87	80	7	14	2	4	4	2		5/5	
	H	1:1000	7/7		2	5	73	80							undil.	6/6	$10^{-0.80}$
		None		3/3	3	10	78	72			Not tested					5/5	
Rabies	I	1:5	4/8	4/4	4	3	53	55	4	3	8	7	35	35	undil.	6/6	$10^{-1.50}$
		None		8/8	4	10	65	65	6	9	2	8	27	18		6/6	

* M.R.[a] = The numerator represents the number of mice which died of virus infection; the denominator the total number of mice injected with virus
M.R.[b] = The numerator represents the number of mice which died of malignant neoplasm; the denominator the number of control mice, or those surviving infection.
N.T. = Not tested.
Ca = Cancer cells.
Hist. = Histiocytes.
Poly. = Polymorphonuclear leukocytes.
Degen. = Degenerated cells.
Karyorr. = Karyorrhexis.

proliferated freely, as indicated by the presence of mitotic divisions (Fig. 2).

Infection with viruses which seemed to multiply in the ascites tumor.—Results of several experiments conducted with Japanese B, St. Louis, louping-ill, and GDVII viruses are summarized in Table 3. The infectious agents were recovered in high concentrations from the ascitic fluids (last column of the table), but, with the exception of four experiments (A, E, F and H), they failed to display an antitumor effect as indicated by the results of bioassay (next to last column). The yields of the virus-infected ascitic fluids were as great as those obtained from the control mice, except for Experiments A, D, F and I. In the latter experiment, as well as in Experiment E, lower percentages of neoplastic cells were observed in smears from virus-injected mice in comparison with controls. This was paralleled in Experiment E by high percentages of histiocytes. A group of the histiocyte cells from a pool of ascitic fluid is illustrated in Figure 3.

Infection with the WS neurotropic strain of influenza A virus (Table 4) was lethal for all mice in Experiments A and B; in Experiment C only a few animals died from virus infection. The yields of the virus-infected fluids were smaller than those of the noninfected control fluids in Experiments A and B. The results of titration of the ascitic fluid (last column of the table) were suggestive of multiplication of the virus in the tumor in Experiments A and B. The results of bioassay and differential cell counts indicated oncolytic action of the virus in Experiment B (1:5 dilution). The remaining results were of equivocal character. It should be noted that ascitic fluids obtained from noninfected control mice in Experiments A and B did not induce tumors in all test animals injected subcutaneously. In Experiment C, the majority of mice surviving virus infection died of ascites tumor. The cell counts failed to indicate a decrease in the number of cancer cells in virus-infected mice. In two instances the results of bioassay indicated an antitumor action of the virus. This was accompanied by higher LD_{50} titer values than in the remaining groups.

Although somewhat similar effects were observed in Experiments A and B with Ilheus virus (Table 5), the results of bioassay in Experiments C and D were indicative of antitumor action of the latter virus. The resulting LD_{50} titers of the virus were also uniformly higher than in the experiments conducted with influenza virus. Morphologic criteria of cell destruction were lacking in Experiments A, B, and C, but in Experiment D an increased number of necrotic tumor cells and the

TABLE 3

EFFECT OF JAPANESE B, ST. LOUIS, LOUPING-ILL, AND MOUSE ENCEPHALOMYELITIS (GDVII) VIRUSES UPON ASCITES TUMOR IN MICE*

Virus	Exp.	Inoculum Dilution of virus suspension	Cause of death Virus infection (M.R.[a])	Cause of death Ascites tumor (M.R.[b])	Mice tapped (no.)	Amt. of fluid aspirated (ml.)	Cell count Per cent Ca Slide I	Ca Slide II	Per cent Hist. Slide I	Hist. Slide II	Per cent Poly. Slide I	Poly. Slide II	Per cent Lymph. Slide I	Lymph. Slide II	Results of transplants† Tumor ratio	Ascitic fluid LD_{50} titer in mice
Jap. B	A	1:2½	6/6		3	6	51	35	7	9	41	45	1	11	1/3	$10^{-4.75}$
		None		5/5	3	11	60	59	15	20	6	9	19	12	5/5	
	B	1:2½	2/5	3/3	2	7	57	81	35	19	4	0	4	0	5/5	$10^{-3.25}$
		1:20	2/5	3/3	2	9	87	79	11	16	1	1	1	4	5/5	$10^{-3.50}$
		None		7/7	2	8	72	86	28	11	0	0	0	3	5/5	
	C	1:5	6/6		1	4	80	74	8	10	9	11	3	5	4/7	$10^{-5.25}$
		None		7/7	1	4	91	67	3	4	4	14	2	5	7/7	
St. Louis	D	1:2½	6/6		3	7	43	35	7	7	40	52	10	6	5/5	$10^{-4.50}$
		None		5/5	3	11	60	59	15	20	6	9	19	12	5/5	
	E	1:5	6/6		2	2	54	50	35	36	5	7	6	7	0/8	$10^{-5.25}$
		None		5/5	1	4	91	81	3	10	4	6	2	3	8/8	
Louping-Ill	F	1:2½	8/8		4	2	60	59	23	10	10	22	7	9	0/4	$10^{-4.50}$
		None		8/8	1	5	46	47	10	10	18	22	26	21	5/5	
	G	1:2½	6/6		3	10	76	72	5	10	15	15	4	3	2/2	$10^{-5.25}$
		None		4/4	2	12	60	59	15	20	6	9	19	12	5/5	
	H	1:10	6/6		1	4	68	66	10	13	16	19	6	2	1/8	$10^{-5.50}$
		None		5/5	3	6	91	67	3	4	4	14	2	5	8/8	
GDVII	I	1:2½	6/6		3	0.5	42	34							3/3	$>10^{-5.00}$
		None		5/5	4	2	65	51			Not tested				5/5	
	J	1:2½	5/5		3	9	77	79	21	20	0	0	2	1	5/5	$10^{-2.50}$
		1:20	5/5		3	9	81	87	16	12	1	0	2	1	5/5	$<10^{-2.00}$
		None		10/10	3	9	82	90	13	10	0	0	5	0	5/5	

* For explanation of abbreviations see footnote, Table 2.
† Only undiluted ascitic fluid used for assay purposes.

TABLE 4

VIRUS (WS NEUROTROPIC STRAIN) UPON ASCITES TUMOR IN MICE*

TABLE 4

EFFECT OF INFLUENZA A VIRUS (WS NEUROTROPIC STRAIN) UPON ASCITES TUMOR IN MICE*

	Inoculum		Cause of death			Amt. of fluid aspirated	Cell Count																						Results of transplants†	Ascitic fluid LD_{50} titer
							Per cent Ca Slide			Per cent Hist. Slide			Per cent Poly. Slide			Per cent Lymph. Slide			Per cent Mitoses Slide			Per cent Degen. Slide			Per cent Karyorr. Slide				
Exp.	No. cells (million)	Dilution of virus suspension	Virus infection (M.R.[a])	Ascites tumor (M.R.[b])	Mice tapped (no.)	(ml.)	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	Tumor ratio	in mice
A	10	1:5	5/5		2	3.0	86	72		5	16		0	1		9	11												2/5	$10^{-4.50}$
		1:10	5/5		3	1.2	71	74		8	9		1	3		20	14												2/5	$10^{-4.00}$
		1:20	5/5		3	2.0	41	36		27	42		9	2		36	20												3/5	$10^{-3.50}$
		None		6/6	3	6.0	80	78		13	8		2	5		5	9												7/10	
B	10	1:5	8/8		3	3.0	4	3		62	51		25	17		9	29												0/4	$10^{-3.80}$
		1:10	8/8		2	3.0	73	74		9	10		8	11		9	5												3/5	$10^{-3.50}$
		1:20	8/8		2	6.0	64	74		10	9		10	15		16	2												2/4	$10^{-3.50}$
		None		8/8	2	9.0	71	63		10	19		7	9		12	9												4/6	
C	12	1:5	1/6	4/5	3‡	6.0	57	70	38	18	27	16	20	2	34	3	1	11	0	0	1	2	0	0	0	0	0		0/4	$10^{-3.60}$
		1:10	2/6	4/4	3	6.0	68	80	76	20	7	14	5	10	7	6	1	2	0	2	1	0	0	0	1	0	0		2/5	$10^{-1.60}$
		1:20	0/5	4/5	3	6.0	89	79	77	6	9	16	2	10	4	1	1	0	2	1	3	0	0	0	0	0	0		2/6	$10^{-1.50}$
		None		8/8	3	6.0	76	76	79	20	16	11	4	4	8	0	2	1	0	2	1	0	0	0	0	0	0		9/10	
	6	1:5	0/6	6/6	3	3.5	73	71	68	17	16	11	5	8	9	5	1	7	0	4	1	0	0	3	0	0	1		4/6	$<10^{-1.00}$
		1:10	1/6	3/5	3	3.0	70	55	18	14	20	30	10	13	29	4	11	20	2	0	2	0	1	1	0	0	0		6/6	$<10^{-1.00}$
		1:20	0/6	2/6	3	2.0	72	61	32	19	31	23	7	6	19	1	0	24	1	2	2	0	0	0	0	0	0		0/4	$10^{-4.60}$
		None		6/7	3	6.0	81	78	80	9	9	6	5	10	10	2	2	2	3	1	2	0	0	0	0	0	0		5/5	

* For explanation of abbreviations see footnote, Table 2.
† Only undiluted ascitic fluids used for assay purposes.
‡ Individual smears made from fluids aspirated from three animals.

TABLE 5

EFFECT OF ILHEUS VIRUS UPON ASCITES TUMOR IN MICE*

	Inoculum		Cause of death			Amt. of fluid aspirated	Cell count																						Results of transplants†	Ascitic fluid LD_{50} titer
							Per cent Ca Slide			Per cent Hist. Slide			Per cent Poly. Slide			Per cent Lymph. Slide			Per cent Mitoses Slide			Per cent Degen. Slide			Per cent Karyorr. Slide				
Exp.	No. tumor cells (million)	Dilution of virus suspension	Virus infection (M.R.[a])	Ascites tumor (M.R.[b])	Mice tapped (no.)	(ml.)	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	Tumor ratio	in mice
A	10	1:5	3/3		2	5.0	78	64		8	16		6	6		8	14												3/4	$10^{-5.75}$
		1:10	3/3		2	7.0	76	84		6	8		9	4		9	4												4/4	$10^{-5.00}$
		1:20	3/3		2	3.0	74	74		13	13		9	9		4	4												0/5	$10^{-4.00}$
		None		4/4	2	10.0	87	80		7	14		2	4		4	2												5/5	
B	10	1:100	4/4		2	2.2	67	83		17	17		8	0		8	0												2/5	$10^{-4.50}$
		1:1000	4/4		2	3.5	86	76		6	11		3	10		5	3												5/5	$10^{-5.50}$
		None		4/4	2	7.5	83	85		8	9		1	4		8	2												5/5	
C	10	1:5	4/4		2	8.0	59	73		17	8		5	17		17	4												0/4	$10^{-6.50}$
		1:20	4/4		2	11.0	76	87		11	7		3	4		10	2												1/4	$10^{-5.00}$
		None		4/4	2	10.0	72	80		14	7		3	3		11	10												4/4	
D	12	1:5	6/6		3‡	5.5	59	56	26	25	7	22	10	18	39	3	16	8	1	1	1	2	1	1	0	1	1		1/2	$10^{-4.60}$
		1:10	5/5		3	5.5	34	64	69	23	20	8	29	29	9	12	3	9	0	1	2	2	2	1	0	0	2		1/5	$10^{-4.40}$
		1:20	4/6	1/2	3	6.0	68	74	40	20	16	26	8	6	13	2	2	4	2	2	3	0	0	14	0	0	0		0/5	$10^{-3.40}$
		None		8/8	3	6.0	76	76	79	20	16	11	4	4	8	0	2	1	0	2	1	0	0	0	0	0	0		9/10	
	6	1:5	6/6		3	2.5	88	12	12	3	62	43	5	8	28	2	9	17	1	1	0	1	8	0	0	0	0		0/4	$10^{-5.50}$
		1:10	6/6		4	1.1	19	38		26	48		9	4		45	8		1	1		0	1		0	0			0/4	$10^{-5.50}$
		1:20	6/6		3	2.0	43	52	6	17	8	34	21	23	34	15	12	21	1	1	2	1	1	3	2	2	0		0/5	$10^{-4.50}$
		None		5/6	3	6.0	81	79	79	9	9	6	6	8	10	2	2	2	2	1	3	0	0	0	0	0	0		6/6	

* For explanation of abbreviations see footnote, Table 2.
† Only undiluted ascitic fluid used for assay purposes.
‡ Individual smears made from fluids aspirated from three animals.

appearance of cells in karyorrhexis (Fig. 4) confirmed to a certain extent the results of the biologic assays.

In Experiments A and B, with West Nile virus (Table 6), the majority of mice which survived virus infection remained ascites-free during a 60-day observation period. In all experiments, the amounts of fluid aspirated from the peritoneal cavity of mice infected with the virus were significantly smaller than those obtained from noninfected controls. The antitumor effect of the virus was indicated by the results of bioassay in Experiments A, B, C and D and unequivocally confirmed in Experiment E, which was conducted on a more quantitative basis. In this experiment, not a single tumor grew from transplants made with dilutions of fluids infected with virus, in marked contrast to the noninfected fluids which gave rise to tumors even when diluted 1:10,000. The biologic findings were not supported by the cytologic observations, since differential cell counts indicated only a negligible decrease in the percentage of tumor cells in virus-infected mice. On the other hand, the strong affinity of the virus for the ascitic fluid was indicated by the results of Experiment D, in which intravenously injected virus was recovered from the ascitic fluid in $10^{-3.75}$ titer.

Six additional experiments, not included in Table 6, were conducted with West Nile virus and gave similar results, including the phenomenon of morphologico-biologic dissociation.

Effect of West Nile virus on ascites tumor implanted in PRI mice.—The PRI strain of mice was observed by Sabin (15) to be genetically resistant to the lethal effect of West Nile virus through interference with its multiplication. Two experiments were therefore conducted in order to study the effect of infection with West Nile virus upon ascites tumors implanted in the PRI strain of mice.

The results of these experiments are summarized in Table 7. The Egypt 101 strain of West Nile

TABLE 6

EFFECT OF WEST NILE VIRUS UPON ASCITES TUMOR IN MICE*

Exp.	Inoculum: Dilution of virus suspension	Cause of death: Virus infection (M.R.[a])	Cause of death: Ascites tumor (M.R.[b])	Mice tapped (no.)	Amt. of fluid aspirated (ml.)	Cell count: Per cent Ca Slide I	Ca Slide II	Per cent Hist. Slide I	Hist. Slide II	Per cent Poly. Slide I	Poly. Slide II	Per cent Lymph. Slide I	Lymph. Slide II	Results of transplants: Dil. of fluid	Tumor ratio	Ascitic fluid LD_{50} titer in mice
A	1:2½	8/10	0/2		Not measured				Not counted							
	1:5	8/10	0/2											Undil.	0/5	
	1:10	8/10	2/2												0/6	
	1:20	4/5	1/1												1/6	
	None		18/18												6/6	
B	1:10	7/7			Not measured	50	90								0/5	$10^{-4.50}$
	1:20	7/7				30	80		Not counted					Undil.	0/4	$10^{-4.60}$
	1:40	3/7	0/4			50	60								0/5	$10^{-4.50}$
	None		7/7			87	80								5/5	
C	1:1¼	6/8	2/2	3	2.0	27	22	54	32	10	22	9	24		0/6	$>10^{-5.00}$
	1:2½	8/8		6	1.0	57	56	15	21	13	8	15	15	Undil.	1/5	$>10^{-5.00}$
	1:5	8/8		8	1.0	20	25	40	57	16	8	34	10		0/5	$>10^{-5.00}$
	None		6/6	4	12.0	57	64	24	18	7	12	12	6		5/6	
D	1:10	8/8		2	4.0	80	72	11	18	1	1	8	9		1/4	$10^{-4.25}$
	1:10†	8/8		2	2.0	84	80	12	16	4	4	0	0	Undil.	1/5	$10^{-3.75}$
	None		10/10	2	7.0	80	90	17	7	1	0	2	3		5/6	
E	1:20	6/6		3	3.0	59	50	26	11	5	34	10	5	10^{-1}	0/4	$10^{-4.50}$
														10^{-2}	0/4	
														10^{-3}	0/4	
														10^{-4}	0/4	
														10^{-5}	0/4	
														10^{-6}	0/4	
	1:100	6/6		2	3.5	44	46	12	8	39	48	5	3	10^{-1}	0/4	$10^{-4.75}$
														10^{-2}	0/4	
														10^{-3}	0/3	
														10^{-4}	0/4	
														10^{-5}	0/4	
														10^{-6}	0/4	
	None		6/6	2	6.0	77	83	6	13	10	1	7	3	10^{-1}	4/4	
														10^{-2}	4/4	
														10^{-3}	4/4	
														10^{-4}	2/4	
														10^{-5}	0/4	
														10^{-6}	0/4	

* For explanation of abbreviations see footnote, Table 2.
† Intravenous inoculation.

virus (12) was injected into 60-day-old PRI mice 4 days after implantation of the ascites tumor cells. The inoculated animals were observed during a period of 2 months, and the number of dead was recorded daily. Every dead mouse was autopsied in order to determine whether it had died of a malignant neoplasm. It may be noted that in Experiment A all noninfected animals died as a result of the ascites tumor within 11–14 days after its implantation. In contrast, only thirteen out of 29 animals given injections of the two concentrations of virus succumbed to the malignant neoplasm. Moreover, death occurred much later in these thirteen animals than in mice in the control group. Infected mice surviving the 60-day observation period were found to be free from ascites and were apparently cured. In experiment B the inoculum consisted of five different concentrations of ascites tumor cells. As in the preceding experiment, the noninfected control mice died of carcinoma within 11–33 days after implantation of the tumor. Inoculation of the virus, however, seemed to interfere greatly with the viability of the tumor, since only five out of a total of 30 mice infected with virus died from ascites tumor. The effectiveness of the oncolytic action of the virus seemed to be somewhat dependent upon the concentration of the malignant cells in the inoculum, as indicated by the death of three out of six mice which had been injected with the highest concentration of inoculum (18 million cells/0.2 ml).

TABLE 7

EFFECT OF WEST NILE* VIRUS UPON ASCITES TUMOR IMPLANTED IN PRI STRAIN OF MICE

Exp.	Inoculum: No. of cells (million)	Inoculum: Dilution of virus suspension	Mortality ratio of mice dying from ascites tumor	Death (days after tumor implantation)
A	23	1:25	6/15	14, 19, 24, 28, 30, 30
		1:100	7/14	14, 18, 23, 23, 29, 53, 57
		None	22/22	14–16
B	18	1:20	3/6	26, 29, 34
		None	8/8	11, 11, 13, 18, 19, 19, 20, 20
	9	1:20	1/6	13
		None	6/6	13, 13, 16, 19, 20, 21
	4½	1:20	1/6	17
		None	6/6	13, 18, 19, 29, 29, 33
	2¼	1:20	0/6	—
		None	5/6	16, 16, 18, 19, 26
	1⅛	1:20	0/6	—
		None	5/6	11, 12, 13, 17, 19

* Egypt 101 strain.

Although the data presented above are based on meager statistical evidence, they seem to lend additional support to the findings that the West Nile virus exercises a biologically discernible interference with the neoplastic process.

Destruction of tumor cells by Bunyamwera virus.—It is not difficult to conclude from the results of the six experiments summarized in Table 8 that Bunyamwera virus exercised a decisive oncolytic effect on the ascites tumor cells. With this virus, in contrast to the previous experiments, the destructive action of the virus upon Ehrlich ascites could be observed whether morphologic or biologic criteria of oncolysis were applied.

In two experiments (A and C) no ascitic fluid could be obtained from mice infected with virus, and their peritoneal cavities were washed with physiologic salt solution in order to provide suspensions of cells for counts, bioassay, and titration for the presence of virus. In the remaining experiments, the amounts of fluid withdrawn from mice infected with virus were very meager in comparison to that obtained from control animals.

The survival of some mice after infection with Bunyamwera virus in Experiments B and C enabled further observation on the ultimate fate of these animals. In Experiment B, out of nine mice withstanding virus infection, three died from ascites tumor. In Experiment C, out of eleven surviving mice, five died from ascites tumors and the others survived.

The differential cell counts gave unequivocal results indicating marked destruction of the tumor cells. The relative number of tumor cells in the infected ascites never exceeded 15 per cent and, in most cases, malignant cells represented one, two, or three per cent of the cell count. This was in striking contrast to the relative numbers of malignant cells, varying from 55 to 93 per cent in fluids collected from noninfected control animals. Figure 5 illustrates a single cancer cell out of several present on a smear in Experiment C (1:5 dilution). The remaining cells shown in this figure are mostly histiocytes and some polymorphonuclear leukocytes.

A more careful observation of morphologic changes in cells following infection with Bunyamwera virus brought out certain details which may assist in an understanding of the mechanism of the oncolytic action of this virus, and in the detection of the existence of such action in viruses which possess it in lesser degree. In Experiment A, the few remaining cancer cells had, in general, a conspicuously necrotic appearance, and it was observed that a large number of the histiocytes present in smears exhibited pronounced phagocytic properties. The assumption would seem to be justifiable that the phagocytized material most

likely represented cellular debris remaining from destroyed cancer cells. In Experiment B, 5 and 15 per cent of cells were counted as malignant elements when 1:10 dilution of virus was used as inoculum. It was observed, however, that these cells were so degenerated that their neoplastic nature could not be conclusively determined by cytologic criteria of malignancy. The above data indicated that oncolysis is probably preceded by induction of cellular degeneration in the neoplastic small to determine accurately the virus content. This was particularly clear from the results of Experiment C in which peritoneal washings seemed to be of very low infectivity for mice injected intracerebrally.

Seven additional experiments were performed along the lines of those summarized in Table 8, and the results gave confirmatory evidence of the oncolytic action of Bunyamwera virus upon the ascites tumor.

TABLE 8

EFFECT OF BUNYAMWERA VIRUS UPON ASCITES TUMOR IN MICE*

Exp.	Inoculum: Dilution of virus suspension	Cause of death: Virus infection (M.R.[a])	Cause of death: Ascites tumor (M.R.[b])	Mice tapped (no.)	Amt. of fluid aspirated (ml.)	Cell count: Per cent Ca Slide I	Ca Slide II	Hist. Slide I	Hist. Slide II	Poly. Slide I	Poly. Slide II	Lymph. Slide I	Lymph. Slide II	Results of transplants† Tumor ratio	Ascitic fluid LD_{50} titer in mice
A	1:$2\frac{1}{2}$	7/7		6	None‡	1	1			Not tested				N.T.	
	None		6/6	3	10.0	78	72							5/5	
B	1:$2\frac{1}{2}$	5/10	3/5	5	0.5	1	1	61	57	2	11	36	31	1/5	$10^{-2.00}$
	1:5	6/8	0/2	7	0.5	3	2	47	31	4	7	46	60	0/5	$10^{-2.50}$
	1:10	6/8	0/2	7	0.5	5	15	60	53	5	8	30	20	0/5	$10^{-2.15}$
	None		8/8	1	5.0	76	93	16	6	4	0	4	1	6/6	
C	1:$1\frac{1}{4}$	3/8	1/5	4	None‡	<1		14		14		72		0/4	$<10^{-2.00}$
	1:$2\frac{1}{2}$	6/8	1/2	2	"	5	11	42	70	3	6	50	16	0/4	$<10^{-2.00}$
	1:5	4/8	3/4	4	"	4	4	47	47	13	7	36	42	0/4	$<10^{-2.00}$
	None		4/4	4	25.0	76	79	18	12	1	6	5	3	5/5	
D	1:$2\frac{1}{2}$	5/5		2	1.6	1	3	9	13	73	65	17	19	0/3	$10^{-4.50}$
	None		4/4	1	5.5	76	62	16	13	3	11	2	11	5/5	
E	1:10	6/6		3	1.2	1	<1	42	64	24	19	33	17	0/1	$10^{-4.75}$
	1:40	6/6		3	1.0	0	2	44	29	32	47	23	22	0/0§	$10^{-4.75}$
	1:160	6/6		3	1.0	7	4	36	39	33	29	24	28	0/0§	$>10^{-5.00}$
	None		5/5	2	10.0	63	73	12	6	7	11	18	10	5/5	
F	1:10	8/8		2	1.0	0	0	84	67	7	12	9	21	0/5	$10^{-2.50}$
	1:250	6/6		2	1.0	0	0	44	70	1	0	55	30	0/5	$10^{-4.50}$
	None		8/8	3	14.0	60	55	4	14	32	28	4	3	4/5	

* For explanation of abbreviations, see footnote, Table 2.
† Only undiluted ascitic fluid used for assay purposes.
‡ Peritoneal washing tested.
§ All of the test mice died of Bunyamwera virus infection.

elements and that the presence of relatively numerous degenerated cells may often be demonstrated in smears in which the number of malignant cells is low, as a result of the oncolytic action of Bunyamwera virus. The various consecutive stages of the morphologic changes terminating ultimately in complete oncolysis due to this virus were discussed and published elsewhere (11).

The results of bioassay were consistent with the morphologic observations. Although in Experiment E most of the animals receiving transplants from mice infected with virus died as a result of this infection, the findings summarized in the remaining experiments indicated again a marked interference of the virus with the neoplastic process.

The LD_{50} titers of the virus did not show the expected uniformity, probably because in most cases the volumes of the ascitic fluids were too

DISCUSSION

An analysis of the experimental results presented in the preceding section leads one to confirm the previously observed factor of "specificity" (8) in the action of viruses in relation to transplantable tumors.

Four of the viruses included in the present study failed in general to parasitize the ascites tumor. In only two experiments out of nine conducted with eastern equine encephalomyelitis, western equine encephalomyelitis, Semliki forest, and rabies viruses, did the ascitic fluid produce a sufficiently high titer to consider the presence of the virus as possible evidence of invasion of the tumor cells. In all other instances, the prompt disappearance of the infectious agents from the ascitic fluid of mice, which ultimately died of viral encephalitis, indicated that the Ehrlich ascites tumors seem to be devoid of certain properties present, for instance,

in the central nervous tissue of mice and which are essential for the attraction and multiplication of these virus particles. The uniformity of this phenomenon, in relation to infection with eastern and western equine encephalomyelitis viruses, leads one to speculate not only upon the lack of attraction of the tumor cells for the virus, but also on the possibility of a positive force of repulsion, almost an antiviral effect.[3] Further studies along these lines are being conducted at present, and some basis for the above theory may be found in the higher multiplication rate of eastern equine encephalomyelitis virus in nonmalignant types of mouse ascites.[4] In connection with the above phenomenon, it should be borne in mind that, out of nine viruses tested, only the eastern virus failed to parasitize Sarcoma 180 tumor (8). However, here the parallel seemed to end, since the same tumor was effectively parasitized by the Semliki forest virus. Conversely, eastern virus parasitized Ridgeway osteogenic sarcoma (8) and was found by Bang and Gey (1) to invade and multiply readily in malignant cells of other origins grown in tissue culture.

It is interesting also to compare the inability of the Semliki forest virus to invade Ehrlich ascites tumor tissue with the alacrity with which it parasitizes the rabbit fibroma and interferes with the neoplastic process (2).

The results of experiments conducted with the Japanese B, GDVII, St. Louis, louping-ill, influenza, and Ilheus viruses must be classified as equivocal. The results of bioassays, the greater frequency of cells in karyorrhexis, and the lower cancer cell counts may perhaps indicate that the effect of Ilheus virus was more persistently oncolytic than the other viruses.

The interfering action of West Nile virus on tumor growth was amply confirmed by the biologic assay technic and by the survival of virus-infected PRI mice (Table 7), but was not reflected in the cytologic observations. It is conceivable that had the experimental animal (Swiss albino mouse) not died of viral encephalitis, actual oncolysis might also have been observed. The differential counts on ascites tumor cells obtained from daily tapping of PRI mice infected with West Nile virus may help to elucidate this phenomenon.

It appeared certain, however, that none of the above-mentioned viruses displayed an oncolytic property similar to that exhibited by the Bunyamwera virus. It may be of interest to note that the louping-ill virus, which exercised a powerful oncolytic effect upon Sarcoma 180 (13), failed to do so with the Ehrlich ascites tumor. This is in contrast to the action of the Bunyamwera virus, which had no effect on the viability of Sarcoma 180 tumor (8), but which destroyed the Ehrlich ascites tumor cells.

The results of the present study indicate that the oncolytic effect seemed to be associated quite specifically with a given virus. For instance, the West Nile, St. Louis, and Japanese B viruses are related immunologically (10), and yet only the West Nile virus was found to exhibit a definite antitumor effect. The singularly powerful oncolysis caused by the Bunyamwera virus also represents a new and unique feature of infection with this pathogen. Viruses which failed to interfere with the viability of the Ehrlich ascites may be found to cause oncolysis of other types of ascites tumors now available and being studied (7), and, if this effect is displayed in a specific way, it may be representative of a characteristic property.

SUMMARY

Studies have been made on the effect of the following twelve viruses upon the Ehrlich ascites tumor: Bunyamwera, eastern and western equine encephalomyelitis, GDVII strain of mouse encephalomyelitis, Ilheus, influenza A (neurotropic WS strain), Japanese B encephalitis, louping-ill, rabies, Semliki forest, St. Louis encephalitis, and West Nile (M 956 and Egypt 101 strains).

By morphologic and biologic criteria, Bunyamwera virus was found to display a powerful inhibitory effect upon the growth of the malignant cells. West Nile virus also interfered with the tumor growth, as indicated by the results of bioassay. Eastern and western encephalomyelitis, Semliki forest, and rabies viruses usually failed to multiply in the ascites tumor, and they had no effect on the viability of the tumor cells. The other six viruses did multiply in the ascites tumor, and their effect on the viability of the tumor cells was either negligible or equivocal. These findings are discussed briefly in the light of the usefulness of the ascites tumor as a medium for viral growth.

ACKNOWLEDGMENTS

The authors acknowledge their indebtedness to Miss Suzanne Voorhies and Mrs. Marion Greene for their invaluable technical assistance.

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Fig. 1.—Smear from noninfected control Ehrlich ascites tumor 9 days after implantation (Table 2, Experiment B, eastern equine encephalomyelitis). Malignant cells predominate. A mitotic figure may be seen.

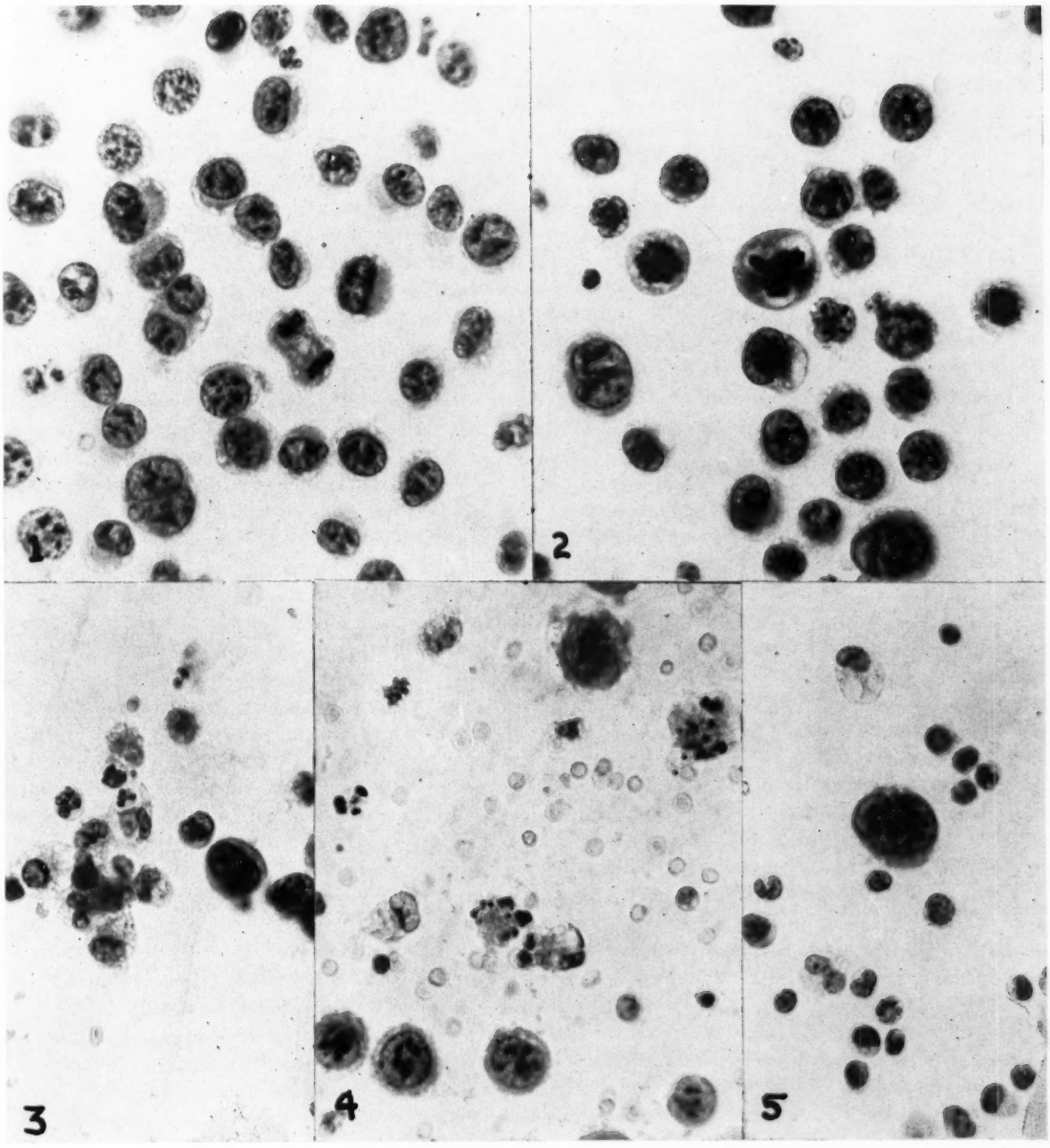
Fig. 2.—Smear from Ehrlich ascites tumor infected with eastern equine encephalomyelitis virus. Malignant cells predominate. A tripolar mitosis is in the center (Table 2, Experiment B).

Fig. 3.—Smear from Ehrlich ascites tumor infected with St. Louis virus. A group of histiocytes is shown in the center. Well preserved malignant cells also may be seen (Table 3, Experiment E).

Fig. 4.—Smear from Ehrlich ascites tumor infected with Ilheus virus. Several degenerated cells showing karyorrhexis and a few well preserved malignant cells may be seen (Table 5, Experiment D, Inoculum: 12 million cells, 1:5 dilution).

Fig. 5.—Smear from Ehrlich ascites tumor infected with Bunyamwera virus. A single cancer cell and numerous inflammatory cells (mostly histiocytes) are shown (Table 8, Experiment 3, 1:5 dilution).

All photomicrographs were obtained with the same magnification (×600).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A Method of Preparing Radioautographs with Adjacent Sections*

TITUS C. EVANS AND WALTER E. MCGINN, JR.

(Department of Radiology and Radiation Research Laboratory, State University of Iowa, Iowa City, Iowa)

The importance of the radioautograph (autoradiograph or autograph) in cancer research is well recognized. Determination of the distribution of radioactive elements in normal and pathologic tissues has been of value in the study, diagnosis, and treatment of various kinds of malignancies. The type of cancer that has been most widely studied in this manner is that of the thyroid. The classic paper of Hamilton, Soley, and Eichorn (19) demonstrated the value of radioautographs as an adjunct to the study of histologic structure of tumors. A disappointing feature of this presentation was that none of the carcinomas studied was able to concentrate the radioiodine. However, Keston, Ball, Frantz, and Palmer (14, 21) reported soon afterward that they had discovered a case in which distant metastases concentrated enough of an administered dose of radioiodine to blacken a photographic plate which had been held against the lesion for a few hours. This exciting report was followed by many studies of thyroid lesions to determine the degree of thyroid function as demonstrated histologically with autographs (6, 12, 13, 15, 18, 22, 24).

The earlier technic of making autographs consisted essentially of placing a section of tissue (mounted on the microscope slide) against a photographic plate for a period of exposure. Afterward, the two were separated and finished independently. In most cases, the shape of the autograph was such that one could determine what general area of the tissue section was responsible. In other instances it was more difficult, and in all cases the resolution was not good enough to permit localization histologically. Resolution and alignment were improved greatly upon the advent of methods in which the thyroid section was mounted permanently with the photographic emulsion (1, 7, 8, 23). These methods involved either coating the tissue section with liquid emulsion, stripping emulsion, or mounting of the tissue section on the surface of a photographic plate. Along with the many advantages of these methods came several difficulties, and these have been covered in the following reviews: Gross and Leblond (18), Boyd (2), Evans (10), and Gorbman (17).

One of the possible sources of artifact in autographs is a chemical action which might falsely register as due to radiation. This is considered in an article by Boyd and Board (4). Errors may also occur in the other direction—such as loss of radioactive material in soluble form. This has been taken into account when necessary by using neutral preservatives or freeze-dry methods as indicated in the reviews (2, 10, 18) and in a recent paper by Holt, Cowing, and Warren (20). Still other methods have recently been introduced either to improve the resolution (11, 16) or to obtain constant registry without permanently adhering the tissue to the emulsion (5).

The method which appears to the writers to be the most satisfactory for study of such sections as those of thyroid tumors is that of permanently mounting the tissue on the photographic emulsion. In order to study a sufficient number of sections at various magnifications, it is advisable that the method not be too difficult or time-consuming, that alignment be accurate, artifacts be recognizable, that histologic detail be observable, and that variations in density of the autograph be subject to measurement. This is a lot to expect in a single method, but through the recent years the present authors have developed a technic which appears to be generally satisfactory for histologic study.

MATERIALS AND METHODS

Preparation of material.—The amount of radioiodine (I^{131}) to be given is judged roughly by several factors. For example, a patient with a thyroid

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tumor estimated at 50 gm. and with an uptake of 20 per cent requires a total thyroidectomy. A dose of 500 μc. is selected. This would yield a concentration of about 2 μc/gm and would give a satisfactory autograph of functioning areas. If the surgeon plans to leave a small piece or an entire lobe, the proposed dose is decreased by about half in order to reduce the possibility of radiation effect on the remaining thyroid. If several days are to elapse before the operation, the proposed dose is increased accordingly (within limits).

Care must be taken to select representative pieces according to the judgment of the surgical pathologist. In addition to the areas under consideration, it is advisable to obtain a small piece of nonpathologic tissue for comparison. The material should be preserved as soon as possible. Carnoy's solution (6 parts absolute alcohol, 1 part glacial acetic acid, and 3 parts chloroform) has been found to be a rapid and satisfactory preservative.[1] The Geiger counter can be used to obtain a preliminary indication of the relative concentration of radioiodine in the various specimens. If the specimen of interest has only weak activity, then speed of preparation becomes an important factor. Maximum speed is obtained by cutting the specimens into small pieces and by applying an intermittent gentle suction during the early part of fixation period. A piece of tissue weighing not over 5 gm. will be preserved in about 5 hours (Carnoy's). From the preservative, one may place the tissue into absolute alcohol (two changes), then into alcohol-xylol, xylol, xylol-paraffin, and finally into two changes of melted paraffin. The tissues are then imbedded in paraffin and sectioned serially through regions of interest.

Preparation of tissue sections.—The tissue is cut at about 8μ. The ribbon is spread on a sheet of paper, and the sections are separated into pairs. If desired, areas of higher radioactivity can be found quickly by scanning with a mica end-window Geiger counter. For diagrams of the following procedures, see Chart 1. The two sections of a pair are separated (*A*), one of these is inverted, and then both are placed on the surface of warm water for spreading (*B*). In order that they spread to the same degree, the two sections should be placed on the warm water (and removed) at approximately the same time. One section is removed on a glass slide and transferred to a pan of cool water; this vessel will later be taken into the photographic darkroom. Aluminum muffin pans, containing twelve cups 1 inch deep and 1¾ inches in diameter have been found to be convenient for this purpose. The identity of each section is marked, with soft blue wax pencil, on the pan above its cup. One pan is usually used for each specimen and may contain several sections for the special method, as well as some extra sections which are later mounted on dental x-ray film for development after different intervals of exposure. As shown at the right of (*C*), the other section of the pair is removed from the warm water with a cover-slip on which it is allowed to dry. The section is placed at one end of the albumin-coated cover-slip, and the other end is labeled. The relative amount of radioactivity of representative sections is determined with a Geiger counter to aid in selecting the proper emulsion, and to give some idea of the exposure time that will be required.

Darkroom technic.—The photographic emulsion to be used is selected according to the degree of localization desired and the relative amount of radioactivity contained in the tissue sections.[2] The following procedures are done in the dark as far away from a red safelight as permits one to see the outline of the section floating on the surface of the water. The slide bearing the emulsion to be used is labeled and is then inserted under the section as shown in (*C*). The section is held in place with a small brush as the slide is removed from the water. The section is placed in the center of the slide as shown on the left in (*D*). The preparation is allowed to drain and is then set aside in a light-proof box to dry. The drying process may be accelerated by use of an air blower. The authors constructed a box with blower attached; and, by the use of baffles and louver boards, the air is allowed to flow freely without admitting light to the slide compartment. After the slides are thoroughly dry, the paraffin is removed with two changes of xylol. The xylol is then allowed to evaporate before the cover-slip preparations are attached. The paraffin is removed from the cover-slip sections (with xylol), and then the final arrangement for the exposure is made. The tissues are cut so that the section appears as a triangle. The cover-slip should be placed over the emulsion of the slide so that the apex of the tissue corresponds to that of the tissue on the emulsion. As shown in (*D*), the orientation of the section will be either one of the two shown at the right. In case of one orientation, the proper position will be obtained by inverting the cover-slip over the slide, in which case the cover-slip is fastened to the left of the section on the emulsion (*E*). If the cover-slip section is in the other position, the cover-slip is to be rotated 180° before being inverted and so comes to lie to the right of the emulsion-section. The cover-slip and slide are held together by small strips of masking tape at the edges.[3] They are then

[1] Bouin's is satisfactory, except that a longer fixation period is required. Gilson's (and others containing bichloride of mercury), if not thoroughly removed before the tissue is placed on the photographic emulsion, will reduce or destroy the autograph. If one is attempting to retain radioactive elements not bound, then the solubility properties of the preserving medium must be considered.

[2] The emulsion found to be most satisfactory (maximum resolution, minimal background fog, and minimal staining of the gelatin) is the Kodak NTB, 25 μ thick, mounted on a 1 × 3 inch glass slide. It is not extremely sensitive and can be used only when the sections contain considerable radioactivity. Kodak Autoradiographic, no screen, is an extremely sensitive emulsion and is to be used with tissues of low activity or when contrast rather than resolution is desired. Kodak Autoradiographic *A* emulsion is also on a microscope slide and is intermediate between the other two in its properties. Lantern slide emulsions, especially that of the 2 × 2 size, are excellent but are somewhat awkward to use in this method, as they are too large for the muffin cups and a bowl is required for the mounting process. In addition, if one desires the autograph mounted on a microscope slide, the emulsion has to be transferred after the hematoxylin staining; this requires care and time. The emulsion should be softened so that it can be separated from the glass with a razor blade and floated off on to the surface of water. It is then transferred to the microscope slide and should be allowed to dry overnight before staining in eosin, dehydrating, clearing, and mounting.

[3] The adhesive strips should not be placed close to the sections. When they are removed later, it should be done very slowly so as to avoid light artifacts.

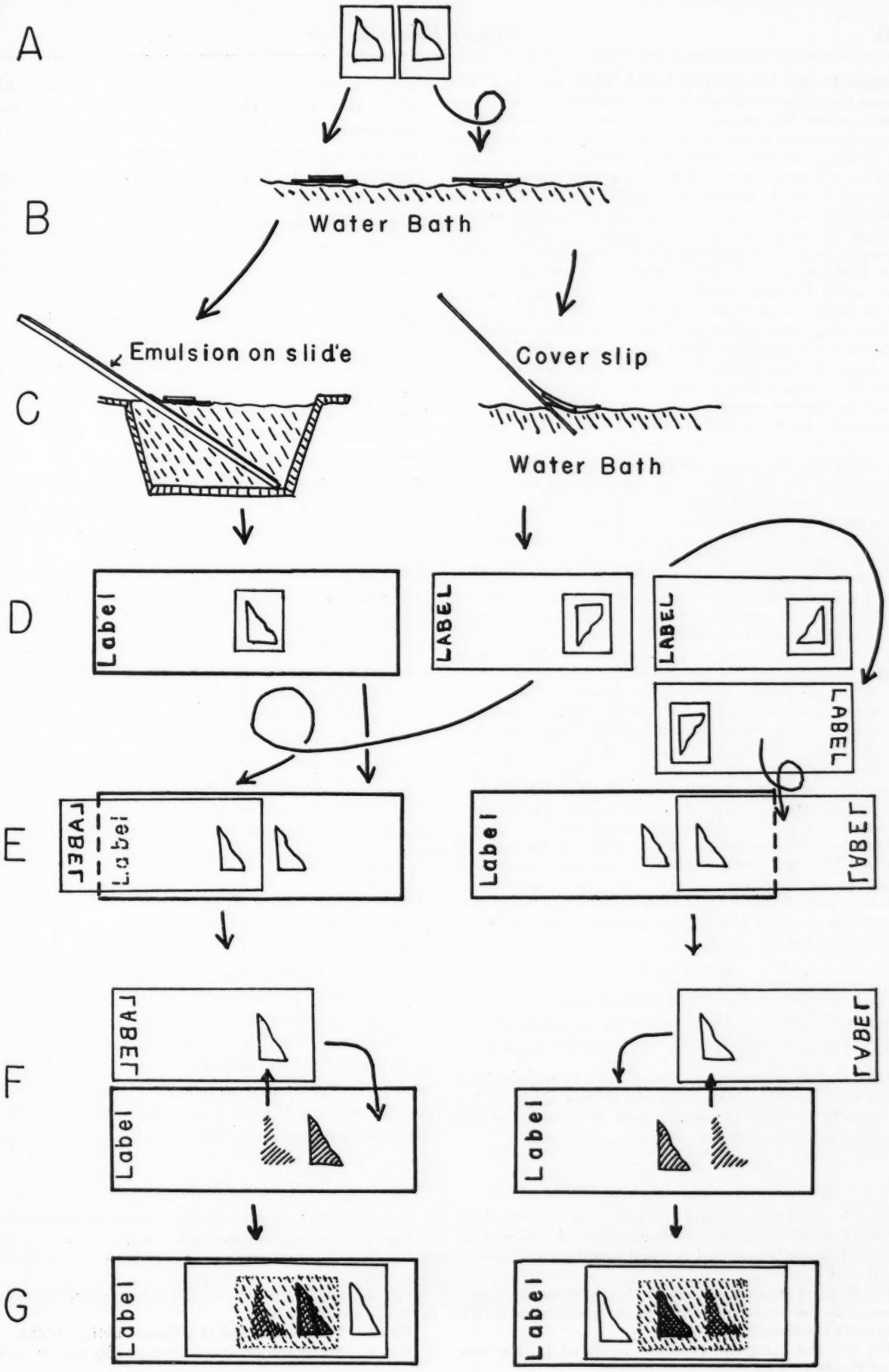


CHART 1.—Illustrations of steps in procedure. See text for details. Steps A, B, and C are described under "*Preparation of tissue sections*"; C, D, and E are described under "*Darkroom technic*"; and G is under "*Final procedures.*"

wrapped in lead foil which is held in place with tape. The preparation is put in a light-proof compartment for the exposure period. The proper exposure time is estimated from the measurements of contained radioactivity and through the use of several sections mounted on dental x-ray film. These films are developed at intervals until the proper exposure period is indicated. At the proper time (usually about a week), the cover-slip is carefully removed, and the autographs are developed. The emulsion is slowly immersed in cold water until thoroughly wet. It is then transferred to the developing solution (Eastman D-11, or D-19) until the image appears. As much of the development as possible is done in total darkness, but sometimes a quick glance in a dim red light is sufficient to show that development is complete or that more time is required. After development, the slide is washed and fixed in acid hypo (Eastman F-5) solution. Some emulsions, especially under the tissue, require a longer time for clearing, and this should be checked very carefully. Usually 15–30 minutes is adequate for the hardening and fixing process. The slides are washed in cold running water for 15–30 minutes and then allowed to dry. The appearance of the autographs in the two different arrangements of the cover-slips is shown in (*F*).

Final procedures.—The sections should be stained as soon as possible after the autographs have been developed, but they may be kept dry for as long as a week without seriously affecting the staining capacity. Although it is not necessary, it is advisable to start with dry slides and place into xylol and then go down through the alcohols to water. Satisfactory results have been obtained with either Delafield's or Harris' hematoxylin, or a mixture thereof. If the tissue is overstained and is then reduced in acid water, care must be taken to avoid too long an exposure. Two strong a treatment at this stage will soften and possibly reticulate the emulsion. Care should be taken to wash well and to follow with alkaline water. The excess emulsion should be removed at this stage when it is soft. A safety razor blade is convenient for this trimming process. After trimming the emulsion, none of it should extend beyond the edge of the cover-slip. The slide bearing the emulsion is then run up to 95 per cent alcohol and counterstained with eosin. The preparation is dehydrated in 95 per cent alcohol, absolute alcohol, alcohol-xylol, and then cleared in xylol.[4] The tissue on the emulsion is then left in xylol while its corresponding section on the cover-slip is stained, dehydrated, and cleared. When both sections are ready, the cover-slip is mounted with balsam (permount, etc.) as shown in (*G*) of the chart.

RESULTS

An example of the final preparation after it is dried and placed on the microscope stage is shown in Figures 1, 2, and 3 with very slight magnification. The arrangement here is that of the right of (*G*) in the chart. The tissue section alone (cover-slip preparation) is shown in Figure 1, and that of the adjacent section mounted on the emulsion in Figure 2, which shows both the tissue and the autograph (directly underneath). Figure 3 is the autograph that was made by the section of Figure 1.

If the three preparations are lined up properly, one can, by the use of a mechanical stage, shift from one to another without much vertical movement. Comparable regions in Figures 1 and 2 can be recognized easily; and with some practice almost any preparation will yield landmarks in the tissue-autograph preparation that can be recognized in the adjacent tissue section. Recognizing areas in the autograph alone is more difficult but is fairly easy at low magnification, and the resolution is not good enough for study at high magnification anyway. A calibrated stage is also helpful in finding the same area in the three preparations. Photographing comparable areas in the three preparations can be done most conveniently by using a photomicrographic camera with a ground glass focusing screen. The objects of interest in one preparation are outlined on the glass with a wax glass-marking pencil, and then the other preparation is moved about until its image fits the outline. Photomicrographs were made of this particular specimen at increasingly higher magnifications to illustrate the ease of localizing the radioactivity at different levels of resolution.

It can be seen even at the low magnification of Figure 1 that this section of the thyroid gland has several nodules containing follicles of different sizes. These follicles appear to be similar in general structure and are well filled with colloid. Yet, as shown in Figure 2 and Figure 3, the uptake is very spotty, and there are large areas that have not taken up the radioiodine at all. In this section, the most generalized activity is in the central and lower left parts of the tissue, and at the periphery of other nodules. The region of particular interest is indicated by the arrow, and it is this area that will be followed at the higher magnifications. As can be seen in Figure 4, the particular nodule of interest (for purposes of describing the method)

[4] Since it is important that all the water be removed, the preparations should be left in absolute alcohol, alcohol-xylol, and xylol for at least 10 minutes each. From three to five changes of xylol are advisable.

FIG. 1.—Photomicrograph, at very low magnification (×6.5), of pathologic specimen of human thyroid. This section is the one that has been turned over and mounted on the cover-slip (H. and E. stain).

FIG. 2.—The adjacent section that has been mounted permanently on the emulsion, showing its autograph immediately underneath the tissue. ×6.5.

FIG. 3.—The autograph that was produced by the section of Figure 1. ×6.5.

FIG. 4.—Section on cover-slip at higher magnification. ×60.

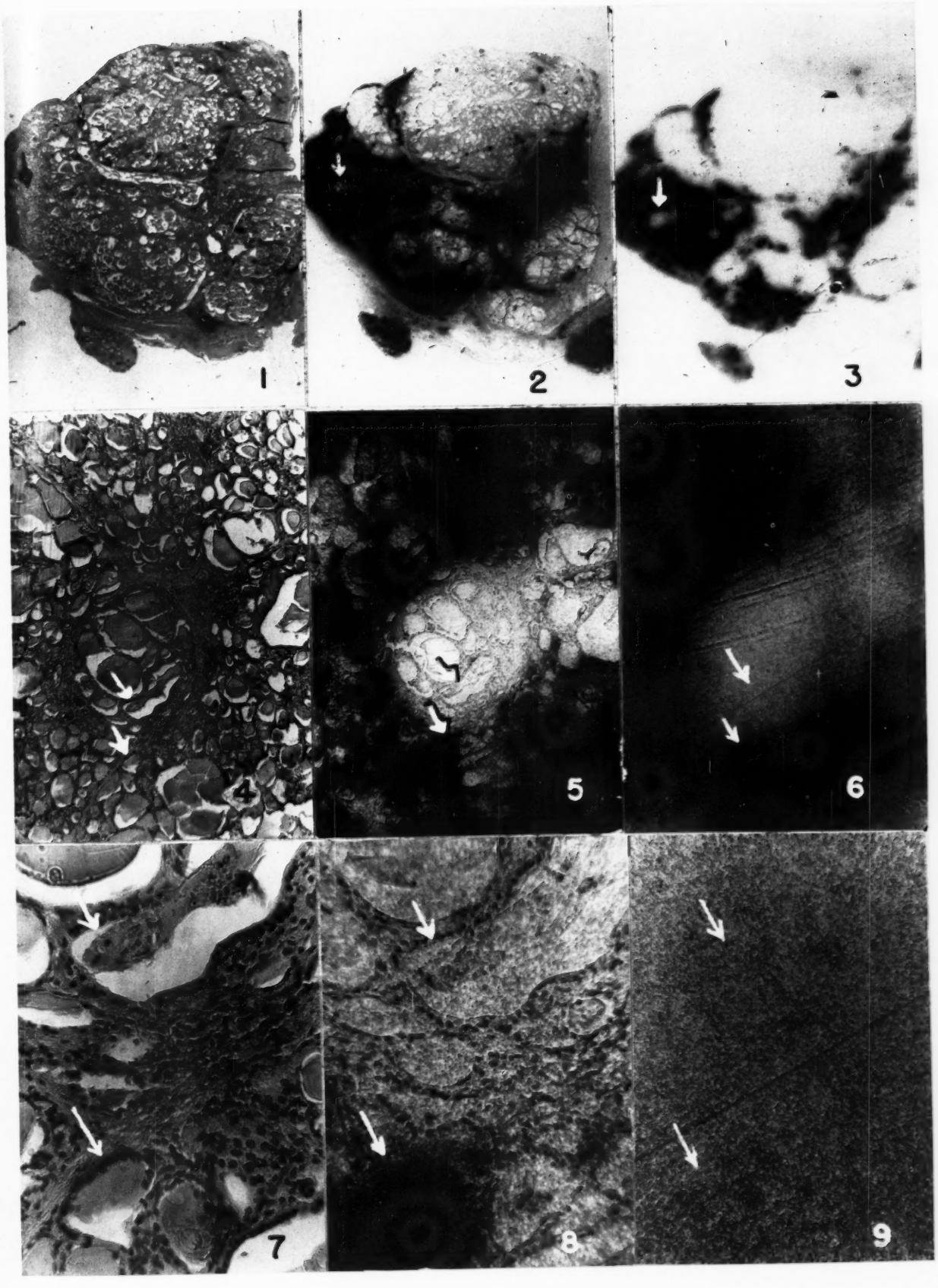
FIG. 5.—Tissue plus autograph of adjacent section. ×60.

FIG. 6.—Autograph produced by section mounted on cover-slip. ×60.

FIG. 7.—Higher magnification of region containing nodule, cover-slip section. ×222.

FIG. 8.—Tissue-autograph of adjacent section. ×222.

FIG. 9.—Autograph produced by cover-slip section. ×222.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contains several follicles surrounded by normal-appearing tissue. The follicles are well filled with colloid, which seems to be similar to that surrounding tissue. The colloid of the adjacent follicles is quite active (Fig. 5), and that in the nodule has no demonstrable radioactivity. In Figure 6, the blank area indicated by the upper arrow coincides with that of Figure 5, showing that the absence of deposited silver underneath is not an artifact due to an air bubble or paraffin or something that might have prevented penetration of the developing solution. In Figure 7, the areas marked in Figures 4, 5, and 6 are shown at higher magnification. The upper follicle which is inactive (as indicated by the results shown in Fig. 8) contains thickened epithelium, and the lower follicle contains rather flat epithelium. In the latter case, the flat epithelium is correlated with a higher iodine uptake, and yet adjacent follicles of similar epithelium give a negative autograph. In Figure 9, the lack of contrast between the active and the inactive areas is shown in the autograph minus the tissue. This is due to the spreading of the radiation at the greater distance between the tissue and the emulsion in the preparation in which the two were placed together only temporarily. The dark lines of Figure 9 are probably artifacts due to brush marks or to fingernail scratches. They do not appear in Figure 8 or in other autographs of the same region.

SUMMARY

A method of preparing radioautographs has been described which consists essentially of utilizing two adjacent sections. One section is inverted on the cover-slip and is also used to produce an autograph by the temporary-mount method. The other section is mounted permanently on the same emulsion. The result is three images in a line on the same slide. These are the tissue alone, the tissue-autograph preparation, and the autograph alone. The advantages of the method are: (*a*) Regions of particular interest in the tissue-autograph preparation can be located easily in the tissue section where the histologic structure may be studied without the interference of the photographic grain which is present in the autograph. (*b*) Possibility of artifact in the tissue-autograph section can be checked by studying the autograph alone. Possible disadvantages are: (*a*) In addition to the inherent limitations of resolution, the structures vary by the thickness of the sections (about 8 μ). (*b*) The technic requires patience and is more time-consuming than more routine methods.

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Demonstration of Antibody in Strain BALB/c Mice to Homologous Tumor S621 by the Use of Two Technics—Anaphylaxis and Tumor Regression*†

MARY A. FINK, G. D. SNELL, AND DIANE KELTON

(Department of Microbiology, University of Colorado Medical School, Denver, Colorado, and Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine)

INTRODUCTION

Various attempts have been made throughout the years to demonstrate the presence of specific immunity to malignant tissue. These studies have involved the use of different species of animals, both random-bred and inbred; and tumors transplanted between species, within species, and among members of inbred strains of a species. Retardation and regression of a second implant of viable tumor have been utilized as criteria of immunity. Such immunity has been induced with normal and maligant tissue, with viable and nonviable tissue, or some tissue components. Published experiments on the induction of immunity to tumor transplants made within an inbred strain (isotransplants) have been recently reviewed by Foley (4).

In this laboratory, during the course of a survey designed to determine the antigenicity of malignant tissues, as evidenced by the production of antibody to a tumor indigenous to an inbred strain, mice of the BALB/c strain have been found to produce antibody to their homologous tumor S621. This can be shown by the classical immunological technic of eliciting an anaphylactic reaction in previously sensitized mice. Further, workers at the Roscoe B. Jackson Laboratory have shown that a second graft of viable S621 will regress in BALB/c mice previously given inoculations of the tumor.

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MATERIALS AND METHODS

The strain of mice used in most experiments was the BALB/c. Three sublines were employed, BALB/cFe, BALB/cSn, and BALB/cJax. The first two of these branched from the third at generations F34 and F37, respectively. Certain tests were also run with strains C57BL/6Sn, C57BL/10Sn, C57BR/aSn, and C57BR/aSn × BALB/cSn hybrids. When indicated, Swiss mice were used to determine the general effect of injections of tumor extracts.

The tumor used in most of the experiments was fibrosarcoma S621. This was induced in a BALB/c mouse, probably line BALB/cJax, approximately at generation F69, by a single injection of methylcholanthrene given April 5, 1948, and was first transplanted July 26, 1948. This means that the tumor arose approximately 32 generations after separation of line BALB/cSn from the Jax subline.

In four of the first five transplant generations the tumor was grown in hybrid hosts with strain BALB/c as one parent. In two cases the noninbred Swiss strain was the other parent. Thereafter, the tumor was grown exclusively in BALB/c's, usually BALB/cSn's but occasionally BALB/cJax's, until transplant 44, made January 27, 1950. In that generation the hosts were of mixed origin (predominantly BALB/c, partly C57BR/cd); and again in generations 77 (transplant date February 2, 1951), 78, and 79 the hosts were BALB/c × C57BL/6 hybrids, in generation 82 BALB/c × CBA hybrids, in generations 84 and 85 BALB/c F_1's (other parent unrecorded), and in generations 86, 92, and 95 through 104 BALB/cFe's.

The first transplant interval was 22 days, but this shortened progressively, dropping to 11 days at the ninth transplant. It now averages 10–11 days.

Our records show 100 per cent "growth" (palpable tumors at the time of the next transplant) in

all hosts through generation 31, and specifically show death from progressive growth of the tumor in generations 2 and 9. Thereafter, the records are less complete, but progressive growth may be presumed to have occurred until generations 84 and 85, when results in four out of seven mice were negative. The reason for these growth failures is unknown; possibly, tissue was taken accidentally from a donor bearing some tumor other than S621. A good line, unquestionably the original tumor, was recovered by using 35-day-old tissue from generation 83. The tumor has since grown without exception in all mice used for routine transplants.

Certain tests were also run with each of three other tumors. There were B1, a mammary gland carcinoma which arose in a C57BL/6Ks mouse in April, 1949 and was in the 69th transplant generation when used; S636, a fibrosarcoma, induced in a C57BL/aSn in December, 1948, and which was in the 100th transplant generation when used; and S653, an undifferentiated rhabdomyosarcoma, induced in a C57BL/10Sn mouse in August, 1949, and which was in the 66th transplant generation when used. In one experiment use was made of an extract of C57BL/6 myeloid leukemia C1498.

The method of preparing and injecting lyophilized tissue has been described elsewhere (6, 7).

In experiments with immunity evoked by inoculation in the tail, the method of Andervont (1) was used. All other tumor inoculations were made subcutaneously by trocar in the right axilla.

Sensitization with lyophilized S621.—BALB/c mice of both sexes, over 4 months old, were used. Animals of this age were selected, because there is evidence that their capacity for production of antibodies is much greater than that of mice 2 months old (3). Mice were injected with lyophilized tumor S621, in one of the following ways:

1. (*a*) The lyophilized tissue was prepared September 30, 1948. It was ground in a cold, sterile mortar in ice to a fine powder, and then ground with sufficient phosphate-buffered 0.85 per cent NaCl, pH 7.1, to make a concentration of 10 mg. tumor (dry weight) per ml. This was allowed to stand overnight, and aliquot portions were stored in a dry ice chest until the time of injection. All the preparations which were tested were shown to be bacteriologically sterile. No attempt was made to demonstrate the absence of a virus.

(*b*) This was identical with the above procedure, except that 2 per cent dried porcine serum (Armour) in buffered saline was used instead of saline alone, to provide an adjuvant effect.

Mice received four intra-abdominal injections of 0.5 ml. each, one every other day for 4 days, representing a total of 20 mg. of lyophilized tumor. An effort was made at all times to use aseptic technic and to maintain a temperature of not over 5° C. when manipulating any wet tumor preparation. Prior to injection, the preparations were warmed at 37° C. in a water bath and used immediately.

2. Lyophilized tumor prepared in 1951 was ground as before, emulsified with Freund's adjuvant (5) prepared from minera oil and Falba. Injections were made subcutaneously in 0.5-ml. amounts, representing 100 mg. of lyophilized tumor. Control mice were injected with Freund's adjuvant alone.

3. Lyophilized tumor prepared in 1951 was ground with buffer as before, to a concentration of 50 mg/ml. This was allowed to stand overnight at 4° C. and was then filtered through gauze and centrifuged for 15 minutes at 3,000 r.p.m. in an International refrigerated centrifuge, model PRl. The supernatant fluid was emulsified in Freund's adjuvant. Mice were given intra-abdominal injections of an amount equivalent to 8 mg. of tumor. One week later, they were given an injection by the same route of a quantity of the extract without adjuvant equivalent to 25 mg. tumor.

Attempts to shock sensitized mice.—At specific intervals following the initial injection (see Tables 1 and 2), shock was attempted by the intravenous injection of varying amounts of tumor S621 extract or extracts of other tissues or tumors as specified for controls. All results presented in Table 1 are from experiments done on mice which had undergone bilateral adrenalectomy[1] at least 2 hours prior to the shocking injection and which were maintained thereafter on drinking water containing 0.85 per cent NaCl. These extracts were prepared in the following manner:

Lyophilized or fresh tumor was thoroughly ground in a cold, sterile mortar with buffered saline, to a concentration of 25 mg/ml. This was allowed to stand overnight at 0–4° C., was then centrifuged at 4° C. as before, and the supernatant fluid, which contained no cellular elements on microscopic examination, was used for injection.

Intravenous injections were made in the tail vein, at a rate of 0.5 ml/min. Preliminary studies of the injection of buffer showed that the animal was physiologically capable of assimilating liquid at this rate without deleterious effect.

Each time the extract was prepared, from two to five Swiss mice and two to three normal BALB/c mice were injected with 0.5 to 1 ml. to demonstrate that the fluid had no general toxic effect. At no time was there an untoward effect from the injection of lyophilized material; one preparation of fresh tumor caused a severe reaction and was rejected. These control injections are tabulated; other control mice recorded in Tables 1 and 2 were injected on the same day, immediately after the experimental ones.

Following the intravenous injection, mice were carefully observed for a period of at least 2 hours. Any symptoms of anaphylaxis (8 and 9), including paralysis, convulsions, gasping, or rapid breathing, were noted; if an animal succumbed, it was autopsied immediately and examined especially for emphysema and hemorrhagic intestines. The time in minutes after injection was recorded for any characteristic finding, and for death.

It is believed that the possibility of subjective error in interpretation was minimized by having two observers, and need not enter in at all when the animal suffered an obvious convulsion, with or without death.

RESULTS

The presence of antibody, as evidenced by sensitization to anaphylactic shock, is seen from the results presented in Tables 1 and 2. Lyophilized S621 was capable of sensitizing BALB/c mice when injected alone, or when injected with porcine serum (Table 1). Anaphylaxis was manifested in these animals subsequent to bilateral adrenalectomy and the intravenous injection of varying amounts of a cell-free extract of either lyophilized

[1] We are indebted to L. W. Wragg for the performance of adrenalectomies.

or fresh S621. Shock was not observed when similar extracts of tumor C1498 and C57BL/6 liver were injected, which attests to the specificity of the reaction. The small number of animals precludes any definitive interpretation of the effect of the amount of tumor used for sensitization, the time interval between injections, or the value of porcine serum as an adjuvant. The amount of tumor injected intravenously, on a dry weight basis, is undoubtedly important, but the exact optimum cannot be ascertained from these data.

When the tumor used for sensitization was incorporated in Freund's adjuvant (Table 2), mice suffered anaphylactic reactions subsequent to the intravenous injection of the tumor extract in the absence of any physiological change wrought by adrenalectomy or surgical trauma. The results indicate that the height of sensitivity was not reached until several weeks after the injection.

Fifteen mice were sensitized by a subcutaneous injection of the tumor extract in Freund's adjuvant, followed in 1 week by an intra-abdominal injection of the extract. Of these, only one animal died of typical convulsive symptoms when injected intravenously at a later time, and four showed moderate symptoms—gasping and prostration—but recovered. Data for these mice are not tabulated.

Three mice, in which two trocar inoculations of S621 had regressed after an original tail inoculation, sustained moderate anaphylactic reactions without death following the intravenous injection of the tumor extract.

Following the observation that extracts of S621 could produce anaphylactic shock in BALB/c mice, it became apparent that some peculiar results were being obtained with this tumor in certain crosses designed to study histocompatibility

TABLE 1

ANAPHYLAXIS IN MICE SENSITIZED WITH LYOPHILIZED TUMOR S621 WITH AND WITHOUT PORCINE SERUM, AND ADRENALECTOMIZED PRIOR TO INTRAVENOUS INJECTION

No. days after sensitization	Sensitizing injection	Shocking injection (ml.)	No. tested	Anaphylaxis*				No. reacting / No. tested
				Severe	Mild	Questionable	Neg.	
12–21	S621	lyophilized S621 0.4–1	12	9	0	2	1	9/12
	S621 (porcine serum)	lyophilized S621 0.5	8	6	2	0	0	8/8
	none	lyophilized S621 0.5–1	15†	0	0	0	15	0/15‡
	S621	lyophilized C1498 0.5	2	0	0	0	2	0/2
	S621	lyophilized C57BL/6 liver 0.5	2	0	0	0	2	0/2
16–21	S621	fresh S621 0.05–0.15	4	0	0	0	4	0/4
		fresh S621 0.25–0.50	3	3	0	0	0	3/3
	S621 (porcine serum)	fresh S621 0.25	3	3	0	0	0	3/3
	none	fresh S621 0.25–0.5	9†	0	0	0	9	0/9

* Criteria for assignment to degree of anaphylaxis:
Severe: convulsion, with or without death (all but one listed above died within 1 hr., 20 min.; slight to marked intestinal congestion, and occasionally mild emphysema noted on autopsy).
Mild: paralysis, spasm or prolonged prostration with or without death.
Questionable: agitation, defecation, urination, gasping, in absence of above symptoms.
Negative: No reaction.

† Including three Swiss mice.

‡ One dead immediately; embolism?

TABLE 2

ANAPHYLAXIS IN MICE SENSITIZED WITH LYOPHILIZED TUMOR S621 INCORPORATED IN FREUND'S ADJUVANT MIXTURE AND NOT ADRENALECTOMIZED PRIOR TO INTRAVENOUS INJECTION

Group	No. days after sensitization	Sensitizing injection	Shocking injection (ml.)	No. tested	Anaphylaxis				No. reacting / No. tested
					Severe	Mild	Questionable	Neg.	
			lyophilized S621						
1	12	S621 in adjuvant	1	10	0	8	0	2	8/10
2	21	S621 in adjuvant	0.25–1	9	3	5	0	1	8/9
3	41	survivors of group 2	0.5 –1	3	3	0	0	0	3/3
4	44	S621 in adjuvant	0.5 –1	4	2	2	0	0	4/4
5	51	survivors of group 4	0.5	2	2	0	0	0	2/2
6	97	S621 in adjuvant	0.5 –1	2	0	2	0	0	2/2
		adjuvant alone	0.5 –1	8	0	0	0	8	0/8
		none	0.5 –1	10	0	0	0	10	0/10

genes. In brief, it may be said that the animals inoculated were of several "isogenic" lines of predominantly pure BALB/c, but each segregating for a single locus (or a single principal locus) causing resistance to S621. Because some of the loci were "weak," it had been found necessary to pretreat the mice to bring out such resistance as was present. Until July 26, 1950, the pretreatment consisted of an intra-abdominal injection of whole blood. Of 23 mice so treated over a 4½-month period, nineteen succumbed to the tumor (expected with one factor for resistance segregating: 17.25 out of 23). On September 8, 1950, the pretreatment was changed to an inoculation in the tail, followed 10–11 days later by removal of the tail and inoculation in the right axilla by trocar. Of 46 mice so treated on September 8, sixteen succumbed (expected: 34.5 out of 46). Subsequently, an additional 117 mice were similarly treated, and 45 succumbed (expected: 87.75 out of 117). Evidently some factor was reducing the fraction of susceptible animals below that expected, and this factor had appeared by September 8, 1950, when the tumor was in the 65th transplant generation.

To shed further light on the problem, experiments were set up to test the possibility of immunizing BALB/cSn, BALB/cJax, and C57BR/aSn × BALB/cSn hybrids against S621 by prior inoculation in the tail. To determine whether any immunity that might be produced was peculiar to this tumor, parallel tests were run with B1 in C57BL/6Sn, with S636 in C57BR/aSn, and with S653 in C57BL/10Sn. It should be emphasized that all experiments involved a tumor tested in its own strain, in which 100 per cent growth is the rule, or in one case in F_1 hybrids which also should be 100 per cent susceptible.

The results are summarized in Table 3, which contains the pooled results of four experiments. It will be seen that a fraction of BALB/c mice and of C57BR/aSn × BALB/cSn hybrids were successfully immunized against S621 by prior inoculations in the tail. The fraction was highest in the case of subline BALB/cSn. In this line, only 44 per cent of the pretreated mice died, as against 100 per cent for the controls. For subline BALB/cJax, the per cent dying was 85, and for C57BR/aSn × BALB/cSn hybrid, 94. With other tumors the mortality was 100 per cent in all cases.

In the last experiment, all survivors were given a second inoculation by trocar 25 days after the first trocar inoculation. None of the BALB/cJax or C57BR/aSn × BALB/cSn hybrid mice survived this second inoculation, but seven of 68 BALB/cSn still proved refractory.

TABLE 3

IMMUNIZATION BY TAIL INOCULATION OF BALB/c AND BALB/c HYBRID HOSTS AGAINST ISOTRANSPLANTS OF S621; FAILURE OF SIMILAR IMMUNIZATION IN OTHER STRAINS

		Fraction dead from trocar inoculation "Immunized" by tail inoculation		
Tumor	Host strain	Dead from first trocar inoculations	Dead from both trocar inoculations	No. dead nonimmunized controls
S621	BALB/cSn	2/16		10/10
		35/68	61/68	20/20
	BALB/cJax	16/20		19/19
		18/20	20/20	20/20
	C57BR/aSn×BALB/cSn	33/35	35/35	21/21
S653	C57BL/10Sn	19/19		20/20
S636	C57BR/aSn	20/20		20/20

Following the method of Kaliss (6), attempts were also made to immunize BALB/cJax mice by injection of lyophilized S621 at various dosage levels. Total dosages per mouse ranged from 0.005 mg. to 50 mg. by multiples of 10. The lyophilized tissue was given intra-abdominally in five injections, and fresh tumors inoculated 10 days after the last injection. There were sixteen to twenty mice in each group. The injections were without effect; the tumor grew in all mice.

DISCUSSION

Because of the many unknown factors operative in the host-tumor relationship, an experimental approach to the demonstration of antibody to malignant tissue is difficult. With almost no knowledge of the identity of the portion of the cell which might be different from normal tissue, and hence antigenic, and with limited knowledge of the type of antibody produced, the classical *in vitro* serological procedures are impractical. Thus, the choice of the highly specific and highly sensitive *in vivo* method of demonstrating antibody by eliciting the hypersensitive reaction known as anaphylaxis, is warranted, especially since this method makes it

possible to determine primarily whether or not antibody *is* produced; and, if it is, to obtain information concerning the portion of the malignant cell which is reactive with antibody, and hence is the antigenic portion.

In defense of the technic of anaphylaxis, it may be said that it is a classical immunological technic, peculiarly applicable in determining the antigenicity of unknown materials. The mouse reacts anaphylactically to tumor antigens in a manner identical to its anaphylactic response to known, purified protein antigens (8 and 9). Previous workers have shown that the symptoms of anaphylaxis in the mouse, while they differ from those in other species, are reproducible and are not dependent on the trauma of intravenous injection or on the general nature of the material injected. In this laboratory, the technic has been tested, both to confirm the work of others, and to become thoroughly familiar with the typical picture of mouse anaphylaxis. Adrenalectomy prior to the shocking injection of antigen renders the animal more sensitive, but the typical shock syndrome may be manifested without employing this means of physiological enhancement to shock. The evidence seems clear that the reaction is due to a *specific* physiologic response incurred as the result of antigen-antibody union.

The anaphylactic response may be elicited with either lyophilized or fresh tumor. This suggests that the ability of lyophilized tumor to sensitize is due to its inherent biologic character, rather than to a change wrought by the process of lyophilization.

It is believed, from the experiments recorded here, that members of the BALB/c strain of inbred mice are capable of forming antibody to their homologous tumor S621. Whether the antibody operative in anaphylaxis is identical with that causing tumor regression is not definitely known. In mice receiving lyophilized tissue previous to the trocar injection of viable tumor, in the one instance recorded here, the implant of tumor was not regressed. But three mice, in which two trocar inoculations of S621 had regressed after an original tail inoculation, sustained moderate anaphylactic reactions without death following the intravenous injection of the tumor extract. It is possible that there is a quantitative relationship between the amount of antibody necessary to sensitize to anaphylactic shock and that necessary to evoke tumor regression.

It is of interest to examine the data to see if any clues can be obtained as to why, in the case of S621, a degree of immunity can be produced against isotransplants, whereas such immunity cannot be produced against the other tumors tested. It should be noted that the capacity for isoimmunization apparently had appeared by September 8, 1950, when the tumor was in the 65th transplant generation. We are unable to say whether it was present prior to this; but the data from the anaphylaxis experiments, in which lyophilized tumor prepared October 30, 1948, sensitized mice to shock, both with itself and with fresh tumor in 1951, suggest that the antigen necessary for isoimmunization was present in the tumor by October 30, 1948.

Several factors acting singly or in combination can be proposed for the observed phenomena of immunization. The antigen may be one inherent in the malignant cell, present either qualitatively or quantitatively in a relationship which remains conducive to histocompatibility unless the conditions for antibody production—age, ability of strain to produce antibody, dosage, etc.—are at their optima.

A second possibility is the occurrence of mutations in the host strain, whereby the hosts become genetically differentiated from the tumor. Since BALB/cJax mice, in which the tumor probably originated, could be less effectively immunized than BALB/cSn mice, differentiation of the host is probably a factor. As already noted, line BALB/cSn separated from line BALB/cJax approximately 32 generations prior to the tumor's origin.

Another possibility is the occurrence of mutations in the tumor. That changes of a mutative nature occur in transplantable tumors during successive transplants is well established. This could be a factor here, but unfortunately we have no specific evidence bearing on this point.

Still another possibility is that change was produced in the tumor by passage through hybrid hosts. Barrett and Deringer (2) have shown that passage of a C3H tumor through C3H × BALB/c or BALB/c × C3H hybrids results in an increased percentage of deaths in inoculated animals of a backcross generation, as compared to the deaths produced by tumor grown only in pure C3H. This establishes an important new fact in regard to transplantable tumors. The situation is not strictly comparable to ours, but S621 was passed through hybrid hosts on several occasions prior to September 8, 1950, and it is possible that the tumor was altered by these passages. Possibly significant in this connection is the observation that C57BR/aSn × BALB/cSn hybrids did not immunize as effectively as pure BALB/cSn's. Unfortunately, the record as to the nature of the hybrid hosts through which S621 had been passed prior to September 8, 1950, is not entirely complete, but it is known that these included BALB/c × Swiss hy-

brids and a hybrid predominantly BALB/c produced by introducing certain C57BR/cd genes onto a BALB/c background.

Finally, the possibility of the presence of a virus in the tumor, established in a host-parasite relationship so balanced that immune substances against the virus successfully prevent tumor growth, has not been ruled out.

SUMMARY

1. Data are presented which demonstrate that inbred mice of the BALB/c strain are capable of producing antibody to a lyophilized preparation of their homologous tumor S621, as evidenced by the *in vivo* immunologic reaction of anaphylactic shock.

2. The state of immunity resulting from tail inoculation of viable S621 is evidenced by the ability of some of the animals to cause regression of a second implant of viable tumor.

3. Possible reasons for the occurrence of these phenomena are discussed.

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Inherited Hormonal Mechanisms and Mammary Cancer In NH Mice and Their Hybrids*

JOHN J. BITTNER

(Division of Cancer Biology, Department of Physiology, University of Minnesota Medical School, Minneapolis 14, Minn.)

That the development of mammary cancer in mice is dependent, in part, upon the hormonal stimulation of the mammary glands has been acknowledged for many years (27). It had also been assumed that ovariectomy of young females of cancerous stocks would greatly reduce the incidence of mammary cancer, until, in 1939, Woolley, Fekete, and Little (17, 36) reported that excision of the ovaries from 1-day-old mice of the dilute brown stock had little, if any, effect in altering the incidence of mammary cancer, although the time of development of the tumors might have been delayed.

These investigators noticed nodular hyperplasia of the adrenal cortex in the ovariectomized females, and, since there was hormonal stimulation of the uterus and development of the mammary glands, they concluded that the adrenals had simulated the function of the ovaries.

The same histological type of adrenal response was seen in ovariectomized females of the cancerous C3H strain (37), whereas mice of the C57BL (37) and the A (16) strains remained in the castrate state.

To determine if mice of various strains are susceptible to spontaneous mammary cancer, females should be observed which have an adequate hormonal stimulation, such as that which results from breeding, and which also possess the mammary tumor agent (5–8), for the incidence of mammary cancer in nonsusceptible animals will be found to be approximately the same as in susceptible mice of most stocks which do not possess the mammary tumor agent.

After females of some sublines of the C57BL stock have been nursed by females of cancerous strains, the incidence of mammary cancer in breeding females may be from 63 to 76 per cent (2, 4, 22). This incidence is approximately the same as that observed in some so-called susceptible mice with the mammary tumor agent (8). When C57 mice with the agent were continued as nonbreeders, 9 and 13 per cent gave rise to mammary tumors (28, 35). Likewise, virgin females of the A strain showed a low incidence, while a high percentage of the breeders developed these tumors (5, 13).

Postcastrational hyperplastic adrenals were found (36–38) in females of strains which showed a high incidence of mammary cancer in virgin females (1, 13, 28, 29, 35), while those strains which showed few tumors in nonbreeders remained in the castrate state (5, 13, 16, 28, 29, 35, 37).

Thus, these observations obtained on ovariectomized females of various stocks suggested a possible association between the adrenal response and the development of, or the ability to induce, mammary cancer in virgin or nonbreeding females of strains which are susceptible to mammary cancer and possess the mammary tumor agent.

Since 1944, numerous reports from this laboratory have been concerned with the development of mammary cancer in virgin females of inbred stocks and their hybrids, associated with various inherited hormonal patterns or mechanisms, as indicated by postcastrational adrenal cortical alterations. In these studies, females of the A strain, with a low incidence in virgins, were crossed with males of stocks showing the adrenal lesions, and the resulting hybrids were continued as either virgins or breeders. The mothers of the A strain would contribute the mammary tumor agent and the inherited susceptibility for mammary cancer; the paternal parents would transmit an inherited hormonal mechanism and, in certain cases, they were also susceptible to mammary cancer.

In the original cross, females of the A stock were mated with males of the Z (C3H) cancerous strain, and the F_1 virgin females were found to have a

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high incidence of mammary cancer, demonstrating that the hormonal pattern which determined a high incidence in the C3H virgin females was transmitted (15, 23). This mechanism has been termed the inherited hormonal influence for mammary cancer (7).

Investigation of an inbred stock in which high incidences of mammary cancer were observed in virgins having the inherited hormonal influence was undertaken to ascertain whether postcastrational adrenal cortical hyperplasia would develop in gonadectomized females and hybrids (7, 15). Females of the A and Z (C3H) strains and their hybrids were ovariectomized. While the castrate females of the A stock showed only slight adrenal changes, there was little or no evidence of hormonal production, as determined by the castrate state of the accessory sex organs (30–32), confirming the observations of Woolley (16). The majority of the Z mice and their hybrids had adrenal lesions with hormonal stimulation of the uterus and mammary glands. Although the castrated mice of the Z stock and their hybrids without the mammary tumor agent showed the adrenal abnormalities, mammary tumors were found only in the females of these groups which possessed the agent. Thus, the tendency for postcastrational adrenal lesions to develop, characteristic of the Z stock, was found to be transmitted as a dominant over the absence of this condition. Also, in these stocks and their hybrids, it was associated with the inherited mammary cancer-inducing hormonal influence (7) for virgin females.

By extending these observations and testing various strains which develop postcastrational adrenal changes, several have been found to have the inherited hormonal influence which induces mammary cancer in virgin females, either in inbred stocks or their hybrids (8–11, 14, 24). Also, by using females of the Z stock as mothers of other hybrids, possible differences between inherited hormonal patterns may be seen as they influence the genesis of mammary cancer in mice.

In 1941, Gardner (21) ovariectomized females of the Strong NH stock (33) and found that thirteen of fifteen animals later developed adrenal tumors and two had mammary cancer. From the condition of the bony pelves and the secondary sex organs, it seemed probable that the adrenal lesions produced estrogen primarily. Confirmatory data were reported by Kirschbaum and his associates (19, 20, 25, 26) for mice of the same stock. In addition (20), spontaneous adrenal tumors were found in intact NH females at 1 year of age, but the development of lesions could be accelerated by removing the ovaries from young mice. Estrogen was shown to be the primary secretion from the altered adrenals.

Because of these observations, mice of the NH stock were tested for the transmission of the inherited hormonal influence which would be expected to produce a high incidence of mammary cancer in $ANHF_1$ virgins (A ♀ × NH ♂). $ZNHF_1$ (Z ♀ × NH ♂) were observed also.

MATERIALS AND METHODS

The three inbred strains used in these experiments were developed by Strong (33, 34). The A and C3H (Z) stocks were separated from Strong's lines at least 20 years ago, while representatives of the NH strain were obtained from Kirschbaum in 1946. As in previous reports, to simplify the designation of the hybrid generations, the C3H will be referred to as the Z stock. All strains have been maintained by brother-to-sister matings.

In Table 1, information is given on how the various hybrids were produced. When females of the A strain were crossed with NH males, the resulting hybrids were called $ANHF_1$. The females of the $ANHF_1$-AxBC and $ANHF_1$-ZbBC generations were derived by crossing the F_1 females with Ax or Zb males, respectively. These males were from lines which did not possess the mammary tumor agent (7).

The $ZNHF_1$ (Z ♀ × NH ♂) females also were mated with either Ax or Zb males, and, for convenience, these hybrids have been called $ZNHF_1$-AxBC or $ZNHF_1$-ZbBC animals, depending upon the paternal parents.

All females received Purina Fox Chow and tap water. Details regarding housing are the same as used for mice of other crosses (8, 12).

EXPERIMENTAL RESULTS AND DISCUSSION

Observations are presented in Table 1 on the development of mammary cancer in virgin and breeding females on inbred stocks and their hybrids, except for mice of the NH stock.

Breeders of the NH stock remained free of mammary cancer when they did not possess the mammary tumor agent. The number living for 10 months, or longer, was 118. Many were not continued after they were removed from the breeding pen, at approximately 1 year of age, while others survived for nearly 2 years. The average age for the entire group was 15 months.

Only preliminary details may be given for NH females which were fostered by females of the cancerous Z stock and for the descendants of these NH mice. In a group of ten, three have had mammary cancer (average age, 271 days), three died

without cancer, and four are living and are between 12 and 15 months of age. Other NH females have been fostered, but they are too young to be included.

Details regarding the other observations will be considered in the discussion.

In virgin female mice the development of mammary cancer has been found to be dependent upon an inherited hormonal mechanism (15, 23), now called the inherited hormonal influence (7), in addition to the inherited susceptibility which in certain stocks and their hybrids has been shown to be associated with postcastrational adrenal cortical hyperplasia (30–32), and the mammary tumor agent (5–8). By testing various strains, this mammary cancer inherited hormonal pattern has been found to be transmitted by, in addition to the Z, the dilute brown (sublines D_2 and D_8), the C and the I stocks. While the females of the I stock have been found to be relatively nonsusceptible to mammary cancer (1, 12), incidences of mammary cancer have been reported in virgin females of the others, which exceeded 50 per cent (3, 8, 13), and ovariectomized females of all strains showed adrenal cortical hyperplasia (14, 18, 24, 36–38).

These results were obtained by the mating of males of the strains being tested with females of the susceptible A strain, where the virgins have an incidence of 4 per cent (5, 14). The lowest incidence of mammary cancer to be observed in the virgin F_1 females was 72 per cent. The average cancer ages for these groups ranged from 451 days (AIF_1) to 555 days (AD_8F_1). In every cross, when the males were representatives of strains with a high incidence in virgin females, their virgin F_1 progeny had a higher incidence than did comparable females of the paternal stock.

Thus, in these virgin F_1 females having maternal parents from the A stock, an incidence of at least 60 per cent would appear to be indicative of the interaction of the inherited hormonal influence, the inherited susceptibility, and the mammary tumor agent in the genesis of mammary cancer in virgin mice.

Since the females of the NH stock showed spontaneous adrenal cortical tumors (25, 26), lesions which could be accelerated by ovariectomy (19–21), males of the NH strain were mated with females of the A stock to determine if they might transmit the mammary cancer hormonal pattern. A high incidence of mammary cancer was not found in the virgins of the $ANHF_1$ generation (10, 11, 14), and even in F_1 breeders, only 43 per cent had tumors (Table 1). In other crosses in which females of the A strain have been used, the incidence in the F_1 breeders has equaled, or exceeded, that seen in breeders of the maternal strain (+80 per cent) (8).

The $ANHF_1$-AxBC (F_1 ♀ × Ax ♂) hybrids showed approximately the same results as did the $ANHF_1$ hybrids, whether they were maintained as virgins or breeders. However, when Zb males, known to transmit the inherited hormonal influence, were crossed with the $ANHF_1$ females, higher incidences of mammary tumors were found in their progeny, in virgins as well as breeders; but the time of appearance of the tumors was not accelerated (Table 1).

TABLE 1

MAMMARY CANCER IN INBRED MICE AND THEIR HYBRIDS

Stock or generation	Cross	Virgin females				Breeding females			
		No.	Cancer (per cent)	Av. age in days		No.	Cancer (per cent)	Av. age in days	
				Cancer	Noncancer			Cancer	Noncancer
$ANHF_1$	A♀ xNH♂	128	16	567	689	68	43	428	617
$ANHF_1$–AxBC	F_1♀ xAx♂	50	14	499	670	40	43	335	533
$ANHF_1$–ZbBC	F_1♀ xZb♂	69	61	508	726	44	82	389	703
$ZNHF_1$	Z♀ xNH♂	62	94	495	639	48	90	345	490
$ZNHF_1$–AxBC	F_1♀ xAx♂	38	30	575	688	30	83	352	652
$ZNHF_1$–ZbBC	F_1♀ xZb♂	31	68	425	663	19	79	261	427
A	A♀ xA♂	111	4	450	573	569	84	330	387
Z(C3H)	Z♀ xZ♂	153	66	404	499	858	95	278	322
NH	NH♀ xNH♂	0				*			

* See text.

Females of the Z stock also were mated with NH males to give $ZNHF_1$ hybrids, in order to study the hormonal patterns transmitted by the parental strains. At least 90 per cent of the $ZNHF_1$ females developed mammary cancer, and a difference of 5 months was seen in the average ages of virgins and breeders (Table 1). Approximately the same incidence noted in breeders of the A stock was found in the hybrids resulting from the cross between the $ZNHF_1$ females and Ax males, and the virgins of this group had a higher incidence than did either the $ANHF_1$ or $ANHF_1$-AxBC virgins. Probably, mammary tumors should have appeared in more of the $ZNHF_1$-ZbBC hybrids, compared to the F_1 population, but the time of appearance of their tumors was comparable to that of the mater-

nal Z stock. In several groups, only a small number could be maintained as virgins, for several other crosses were being observed concurrently.

Hybrids of seven crosses have been observed where females of the Z stock have been mated with males of other strains. In virgins of these F_1 groups, the lowest incidence and latest cancer age were found in the hybrids with fathers of the A strain, a stock with a low incidence of mammary cancer in virgins, whose breeders are susceptible to mammary cancer (5) and whose ovariectomized females remain in the castrate state (30–32). The earliest average cancer age, among both F_1 virgins and breeders, and a high incidence occurred in the ZI cross, when males of a nonsusceptible strain were tested (1, 12). With the exception of the ZAF_1 group, the $ZNHF_1$ virgins had the latest tumor age, and, in the breeders, it was 1.5 months later than that observed for the ZAF_1 breeders.

These data might indicate that the inherited hormonal pattern transmitted by mice of the NH strain could, at this time, be considered as distinct from that which has been called the mammary cancer-inducing inherited hormonal influence for virgin females (7). Instead of producing a high incidence of mammary cancer in virgin animals which are susceptible and have the mammary tumor agent, such as the $ANHF_1$ hybrids, evidence was obtained in the $ANHF_1$ and back-cross hybrids that the hormonal mechanism characteristic of the NH stock tends either to inhibit or to delay the time of development of these tumors, not only in virgin females but also in breeders. The inherited hormonal influence for mammary cancer in virgin females, as possessed by the Z stock, was found to be dominant over the hormonal mechanism transmitted by the NH mice, although some effect of the NH pattern may be apparent in influencing the average cancer age in the hybrids.

Before it will be possible to establish definitely the influence of various inherited hormonal patterns on the genesis of mam ıary cancer in mice, consideration must be given to the possibility that the other causative factors for mammary cancer may interact to produce the observed results. Previous reports (7–14), and others reviewed in (8), have shown that, when mice of various cancerous strains were mated, the reciprocal hybrids may not show comparable incidences and average cancer ages. These data suggested that the mammary tumor agents transferred by mice of different cancerous stocks did not have the same tumor-inducing activity in reciprocal hybrids possessing the same genetic constitution for the development of spontaneous mammary cancer. In some crosses, the percentage of the F_1 virgins, derived by crossing two cancerous stocks, to have mammary cancer was only slightly higher than that observed in the $ANHF_1$ virgin females. However, while the $ANHF_1$ breeders continued to have a comparatively low incidence (Table 1), high incidences were observed in the F_1 breeders of the other series ([8] and unpublished data).

Some influence of a possible difference in the activity of the mammary tumor agent in the hybrids of the NH mice may be apparent in the following data, although the numbers are small and some animals are still under observation.

A few of the first and second generation descendants of the fostered NH females, which obtained the mammary tumor agent by nursing females of the cancerous Z stock, were mated with Zb males. Preliminary data may be cited on the development of mammary cancer in their F_1 hybrids, which were maintained as breeders to study the propagation and transmission of the agent in NH mice. No difference has been found in the cancer incidence in the hybrids, which can be related to the generation of the NH mothers, and the youngest living animals are 9 months of age. In depleted litters, thirteen (100 per cent) have had mammary tumors at an average age of 293 days. In undepleted litters, fifteen have had tumors (average age, 276 days), ten are living, and one succumbed without cancer at 330 days of age. Thus, of these F_1 females with mothers of the NH stock with the agent, 28 of 29 (97 per cent) which have died to date have had mammary cancer. While the results may be altered after all the animals have died, there is considerable difference, even in depleted litters, between the average cancer age and that seen in the $ZNHF_1$ breeders (Table 1).

Thus, the activity of the mammary tumor agent must be given some consideration in the interpretation of data on the genesis of mammary cancer in mice, for the agent transferred by females of a cancerous strain may have different tumor-inducing properties when it is propagated and transferred by animals of a different genetic constitution.

SUMMARY

A small number of fostered NH females with the mammary tumor agent were observed, and a few developed spontaneous mammary cancer.

Since mice of the NH strain develop spontaneous and postcastrational adrenal cortical alterations, they were tested to determine if they might transmit the mammary cancer inherited hormonal influence, which induces mammary cancer in virgin females that are susceptible and have the mammary tumor agent.

When females of the A strain, with a low incidence in virgin females, were mated with NH

males, the development of mammary cancer in the $ANHF_1$ hybrids, even in breeders, was either delayed or inhibited, possibly because of the action of the NH hormonal mechanism.

By mating NH males with females of the Z stock, known to transmit the mammary cancer inherited hormonal influence, a high incidence of mammary cancer was observed in the $ZNHF_1$ virgin females.

By mating the $ANHF_1$ females with males of the Z stock, an increased incidence was seen in the progeny when they were kept as nonbreeders.

Other observations were reported upon the development of mammary cancer in back-cross animals of different genetic constitutions.

While the data suggested that the hormonal pattern transmitted by the NH mice may have an inhibitory effect on the development of mammary cancer, consideration must be given to the interaction of the other causative factors, together with possibly different inherited hormonal mechanisms.

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Influence of Postirradiation Thymectomy and of Thymic Implants on Lymphoid Tumor Incidence in C57BL Mice*

HENRY S. KAPLAN, MARY B. BROWN, AND JANICE PAULL

(*Department of Radiology, Stanford University School of Medicine, San Francisco, Calif.*)

In several strains of mice, spontaneous and induced lymphoid tumors exhibit a tendency to arise in the thymus gland. Furth and his associates (1, 8) found that the incidence of spontaneous lymphomas in a susceptible strain was sharply reduced after thymectomy. Subsequently, Kaplan (4) and Law and Miller (6, 7) reported a similar influence of thymectomy on lymphoma development under other experimental conditions. Furth (1) and Kaplan (4) attributed this phenomenon simply to removal of the tissue of maximal susceptibility. However, Law and Miller (6, 7) reported that the incidence of lymphoid tumors could be restored to essentially normal levels in thymectomized mice by autologous or homologous subcutaneous thymic implants, suggesting that the thymus may be involved in the pathogenesis of the disease in an additional role, possibly of endocrine nature.

In all these investigations, thymectomy was performed before exposure to the tumor-inciting agent, or, in the spontaneous lymphoma studies, at a time when the endogenous agents were presumably still active. Moreover, Law and Miller implanted their thymic grafts prior to the start of carcinogen application; thymic tissue was therefore present in the animal during exposure.

It seemed of interest to study the effect on lymphoid tumor incidence of thymectomy at varying intervals after irradiation and to determine whether homologous thymic tissue implanted after irradiation can restore the lymphoma susceptibility of thymectomized mice. This paper is concerned with two such experiments.

MATERIALS AND METHODS

Strain C57BL mice of both sexes, aged 33 ± 3 days at the start of irradiation, were used in both experiments. Littermates were distributed at random among the various experimental groups. The animals were identically caged and had free access to Purina Laboratory Chow and water. Animals that died following operation or irradiation, or were too autolyzed to permit autopsy diagnosis, have been considered indeterminate. Except for animals dying with grossly typical lymphomas, tissues taken at autopsy were routinely subjected to histological examination.

In Experiment I, eight groups of sixteen mice each were treated with four total-body x-ray exposures of 168 r at intervals of 8 days.[1] One remained intact and served as an irradiated control group. The others were designated for thymectomy at 4, 6, 8, 10, 12, and 16 weeks, respectively, after the last irradiation; an additional group of irradiated "spares" was assigned to the 6-week group when operative deaths in this group became excessive. The technic of thymectomy was identical with that previously described (4). The freshly removed thymus glands were minced and suspended in 0.6 cc. of ice-cold Locke's solution; 0.2 cc. of this thick suspension was then injected intraperitoneally into each of three normal C57BL recipient mice about 1 month old as a bioassay for the presence of lymphoid tumor cells (2). The surgical survivors and bioassay recipients were then maintained without further treatment and were observed for the development of lymphoid tumors. When it became apparent that many thymus glands removed 12 weeks after irradiation contained obvious tumors, the group designated for thymectomy at 16 weeks was sacrificed and autopsied instead; representative tissues were studied histologically to determine the number of lymphoid tumors present at that time (137 days after the start of irradiation).

In Experiment II, seven groups, each containing 50–60 mice, were employed. All but one of these received total-body irradiation in four doses of 168 r at 4-day intervals.[1] Four groups were thymectomized by the usual technic (4); the other three were subjected to a sham operation in which the thorax was opened but the thymus was left intact. All operations were performed 4 days before the start of irradiation. Whole thymus glands were quickly excised from donor C57BL mice 33 ± 3 days of age; some were then rapidly given a single x-ray dose of 990 r *in vitro*, after which one entire irradiated or nonirradiated thymus was implanted through a small cutaneous incision into the axillary subcutaneous tissue of each mouse of the recipient groups within 1–2 hours after the last total-body x-ray exposure. The animals were then observed for the appearance of lymphoid tumors.

RESULTS

The results of Experiment I, as tabulated about 12 months after the start of irradiation, are indicated in Table 1. Tumor incidence in the irradiated control group reached 100 per cent at 10 months. No tumors occurred in animals thymec-

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[1] Physical factors were: 120 kvP; 9 ma.; 0.25 mm. Cu + 1.0 mm. Al added filter; 30 cm. mouse-target distance; 32 r/min.; HVL = 0.39 mm. Cu.

tomized 4 weeks after irradiation. Thymic lymphomas were encountered sporadically as early as 6–8 weeks after irradiation. They were present in the excised thymuses of one-third of animals operated upon at 10 weeks and in over one-half of those thymectomized at 12 weeks.

Thymectomy proved to be a strikingly effective prophylactic measure. Excluding animals that already had lymphomas at the time of surgery, all other survivors, even those thymectomized as late as 12 weeks after irradiation, have remained completely free of lymphoid tumors. Moreover, thymectomy appeared to be of therapeutic value in a small number of mice, presumably as a result of fortuitous removal of an early lymphoma still confined to the gland. In two instances, the excised thymus yielded a positive bioassay, establishing the presence of tumor within it, but no evidence of recurrent or disseminated lymphoma has appeared in the donor mice during a postoperative observation period of over 9 months. In addition to these two apparent "cures," prolonged postoperative survivals of from 6 to 12 weeks or more have occurred in at least three other mice found to have a gross thymic tumor at operation. In these three instances, residual tumor ultimately disseminated to cause death, however. Since the bioassay method proved to be quite insensitive, and in several instances failed to yield growth of well established tumors, it seems probable that there were other, undetected "cures" following thymectomy.

One group of 24 mice was sacrificed, in lieu of thymectomy, at experimental day 137, 16 weeks after their last irradiation, to obtain data on the incidence of lymphoid tumors at this time. Only ten appeared on gross inspection to have a definite or probable thymic lymphoma, but 23 (96 per cent) were positive on histologic examination. At least three of these lymphomas were so early that they were still confined entirely to one thymic lobe, confirming similar instances reported earlier (3). More recently, in another group of similarly irradiated mice killed at day 120 for biochemical studies, over 80 per cent were found to have lymphoid tumors in the thymus, several of which were still limited to one lobe. In some instances, the tumor cells had not yet completely replaced the normal architecture, and it appeared that involvement of the medulla was incomplete, suggesting that the disease first appears in the cortex of the gland.

The results of Experiment II, which was terminated after about 20 months, are summarized in Table 2. Tumor incidence in all the sham-operated groups exceeded 90 per cent, and was not affected by the presence or absence of an exogenous thymic implant. In striking contrast, there was only one lymphoma among 31 determinate mice (3.2 per cent) in Group III, which received no implant after thymectomy and irradiation, and a similarly low incidence (within the range of spontaneous lymphoma incidence for the strain) in nonirradiated thymectomized Group IV animals which received an irradiated thymic implant. Of particular interest, however, is the occurrence of twelve lymphomas among 31 determinate mice of Group I (38.7 per cent) and of eight lymphomas among 39 determinate animals of Group II (23.1 per cent), which received nonirradiated or irradiated thymic implants, respectively, subsequent to thymectomy and systemic irradiation. Statistical comparison with the results in Group III is complicated by the different rates of operative, post-irradiation, and miscellaneous deaths.

If we calculate χ^2 (with 6 degrees of freedom) for Groups I, II, and III in Table 2, using the total number of determinate mice without reference to the presence of tumors, we are testing the hypothesis that the three treatments (I, II, III) are equivalent with respect to incidence of various causes of

TABLE 1

EFFECT OF THYMECTOMY AT INTERVALS AFTER IRRADIATION ON DEVELOPMENT OF LYMPHOID TUMORS IN C57BL MICE

Group	Thymectomy (weeks after last x-ray)	Initial no. mice	Operative deaths	Net no. mice	Lymphoid tumors* No.	Lymphoid tumors* Per cent	No. recipients (bioassay)	Lymphoid tumors in recipients
I	none	16		16	16	100		
II	4	16	4	12	0	0	48	0
III	6	24	6	18	2	11	72	1
IV	8	16	4	12	1†	8	45	0
V	10	15	7	8	3	38	45	4
VI	12	16	3	13	7	54	48	8

* Based either on result of bioassay or ultimate death of operated mice with recurrent or disseminated tumor.

† One mouse died with a thymic lymphoma 2 weeks before time for thymectomy.

indeterminacy. The value of χ^2 is 8.062,[2] which is not significant, in that the probability of a larger value of χ^2_6 is about 0.25, and we find no reason for regarding the treatments as different in this respect.

If then we calculate χ^2 for the table with determinate mice subdivided into tumor and nontumor, we obtain a value of χ^2 (with 8 degrees of freedom) equal to 18.685. This is significant at the level 0.02, and we conclude that the treatments *do* differ as to effect on tumor incidence. It is clear that the difference arises in the contrast between Group III and the other two groups on the tumor versus nontumor breakdown.

The histologic appearance of the uninvolved and tumor-infiltrated thymic implants was essentially similar to that described by Law and Miller (7). The implants were involved by tumor to about the same degree and with roughly the same frequency as the superficial lymph nodes, and distinctly less often than the abdominal or thoracic viscera. Lymphomas had in most instances replaced the mediastinal structures, even in the thymectomized animals of Groups I and II. Incomplete removal and subsequent regeneration of the thymus may have been responsible for the appearance of some of the tumors in these groups. However, there was no recognizable factor of selection in the experimental design which would have contributed to a greater frequency of incomplete operations in these groups as compared to Group III. The fact that some thymic implants were plainly unaffected by tumor in animals with disseminated lymphomas confirms the observation of Law and Miller that the tumors do not originate in the implants (Fig. 1).

It is of interest that the latent period for lymphoid tumor development exhibited a much greater range in Groups I and II than in the highly susceptible sham-operated groups or the refractory Group III animals. Although it is reasonable to suspect that the earliest tumors in Groups I and II occurred in incompletely thymectomized mice, the considerable number of late tumors cannot be explained on this basis. All the late tumors were lymphocytic lymphomas; none of the reticulum-cell and Hodgkin's-like tumors to which aged mice of this strain are prone were included as lymphomas in any group.

DISCUSSION

The design of Experiment II was distinctly more stringent than the experiments reported by Law and Miller (6, 7) in that thymic implants were not introduced until exposure to the inciting agent (x-rays) had been completed. Perhaps because the experimental conditions were more rigorous, thymic implants failed to restore lymphoma incidence to the level of sham-operated irradiated controls, but they did nonetheless significantly augment susceptibility. The mechanism whereby thymic implants exert this influence can only be conjectured. The thymus has for so many years frustrated the efforts of investigators seeking to ascribe endocrine activity to it that such an interpretation, though perennially attractive, seems premature at this time. It would seem, however, that the thymus plays a dual role in lymphoma pathogenesis; it is the foremost site of origin for such tumors in certain strains of mice, and its presence appears to condition susceptibility, independently of the site of tumor origin, in all strains thus far tested.

Moreover, as indicated by Experiment I, removal of the thymus as late as 12 weeks after irradiation effectively prevents subsequent lymphoma development in C57BL mice. It is likely that the main effect of thymectomy in this strain is the removal of the tissue of maximal susceptibility, but the indirect thymic influence probably plays some part as well. That this indirect mechanism may be radiosensitive is suggested by the lesser

TABLE 2

EFFECT OF THYMIC IMPLANTS ON DEVELOPMENT OF LYMPHOID TUMORS IN THYMECTOMIZED AND IRRADIATED C57BL MICE

Group	Operation	Irradiation		Initial no. mice	Operative deaths	Radiation deaths	Other losses	Total	Determinate mice		
		Host*	Thymic implant†						No tumor	Lymphoid tumor	Per cent
I	Thymectomy	yes	no	64	15	10	8	31	19	12	38.7
II	Thymectomy	yes	yes	64	13	7	5	39	30	9	23.1
III	Thymectomy	yes		64	8	14	11	31	30	1	3.2
IV	Thymectomy	no	yes	64	11		7	46	44	2	4.3
V	Sham	yes		65	10	2	1	52	4	48	92.3
VI	Sham	yes	yes	65	12	6	0	47	3	44	93.6
VII	Sham	yes	no	64	7	5	8	44	2	42	95.4

* Four doses of 168 r at 4-day intervals.
† 990 r *in vitro*.

[2] The statistical analyses were kindly performed by Dr. Lincoln Moses, Assistant Professor of Statistics and Public Health, Stanford University.

effectiveness of thymic implants pre-irradiated *in vitro* (Group II, Experiment II).

The data of Experiment I help to establish parameters with respect to the time of lymphoid tumor onset in irradiated C57BL mice. As previously noted (3), tumors are present sporadically in the thymus as early as 6 weeks after the end of irradiation (experimental day 66), attaining an incidence of 96 per cent at day 137. Under conditions of maximal susceptibility, it would appear that the factors responsible for lymphoid tumor development are operative for a relatively brief period of the order of a few weeks or less. Moreover, collateral evidence (5) suggests that the tumorigenic process may be reversible during much of this time. More intensive and detailed investigation of the altered morphologic and functional interrelationships of the lymphoid tissues and endocrine organs during this critical time period therefore seems indicated.

SUMMARY

Two experiments concerning the role of the thymus in lymphoid tumor genesis in irradiated C57BL mice are reported. In Experiment I, thymectomy as late as 12 weeks after irradiation completely suppressed subsequent lymphoid tumor development and effected apparent cures of two mice whose excised thymuses were shown by bioassay to contain tumor. Tumors were encountered sporadically in thymuses removed at 6 weeks, in about one-third of those removed at 10 weeks, and in the majority of those removed 12 weeks after irradiation.

In Experiment II, thymectomized mice implanted after irradiation with homologous thymic grafts, either intact or irradiated *in vitro*, had a significantly higher incidence of lymphoid tumors than litter-mates similarly treated but receiving no thymic implants. Incidence remained far below the levels of sham-operated, irradiated groups, and was lower for the irradiated than for the intact implants.

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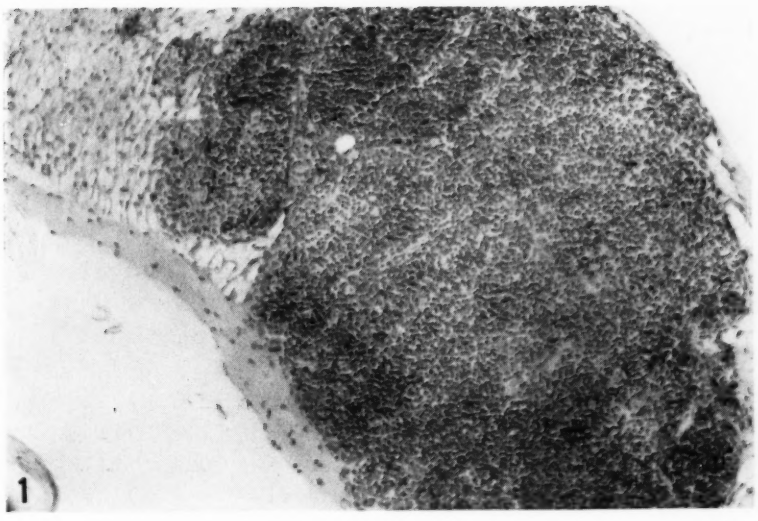
Fig. 1.—Subcutaneous thymic implant, still exhibiting cortical and medullary zones and no evidence of lymphoid tumor infiltration. This implant was removed from a sham-operated, irradiated animal which died with a mediastinal thymic lymphoma 165 days after the start of the experiment.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The Induction of the Shope Papilloma in Homologous Transplants of Embryonic Rat Skin*

HARRY S. N. GREENE

(Department of Pathology, Yale University School of Medicine, New Haven, Conn.)

The induction of the Shope papilloma in embryonic rabbit skin infected *in vitro* and transplanted to the brains of adult rabbits has been described (2). Mice, rats, and guinea pigs as well as rabbits were used as hosts for the infected embryonic tissue, and papillomas were induced as readily in the resistant species as in homologous animals. The suggestion, implicit in this finding, that the response to infection was a function of local cellular factors rather than of the constitution of the host was subjected to further investigation, and one phase of the study is reported in the present paper. The experiments to be described are concerned with attempts to determine whether the factors governing infectability or response to infection are inherent in cells or represent developmental acquisitions. The method of approach consisted of treating embryonic skin derived from resistant species at different stages of development with the Shope papilloma virus and observing the fate of the tissue following transfer to the brains of homologous hosts.

MATERIALS AND METHODS

Guinea pigs, mice, rats, and hamsters were used in these experiments. These species are known to be resistant to infection with the Shope papilloma virus, and control groups inoculated with active material showed no evidence of response on either gross or histologic study.

Embryos were obtained at different periods of gestation, and the skin was dissected under sterile conditions. After infiltration with virus fluid, fragments of epidermis measuring approximately ½ mm. in diameter were transplanted to the brains of adult animals of the same species. The technics used in infiltrating the skin and in transplantation to the brain have been described (1). The recipient hosts were killed 29–31 days after transfer and the transplants removed for histological study.

RESULTS

The skin of rat embryos proved susceptible to infection with the Shope papilloma virus, and transplants contained typical papillomatous lesions. In contrast, the skin of guinea pig, mouse, and hamster embryos subjected to the same treatment showed no changes comparable to the reaction observed in the rabbit.

Rat embryonic skin.—The embryos were derived from 32 rats killed at various stages of gestation. The date of mating was not determined, and the crown-rump length of the embryos rather than age was used to indicate the extent of development. Individual groups of embryos measured from 0.4 to 3.8 cm. in length and included representatives of different stages of development from early to late gestation. The skin of very early embryos was not dissected, and the entire embryo was transplanted after treatment with virus fluid. Six to ten rats were used in each experiment, and the animals were killed 1 month after transfer.

The transplanted skin invariably survived and grew irrespective of the age of the embryo. In several instances, no abnormalities were noted on histological study of the transplants derived from a particular group of embryos, but the fact that typical lesions were found on repeated transfer of skin of approximately the same embryonic age suggests that the failure was due to the use of a noninfectious virus or to some fault in procedure rather than to resistance to infection. With these few exceptions, typical papillomatous lesions developed in 50 per cent or more of the transplants derived from individual groups of embryos regardless of age. The incidence of papillomatous transformation was highest in skin transplants derived from embryos 1–2 cm. in length and tended to decrease in younger and older age groups. It should

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be noted, however, that the difficulties associated with the handling of the thin, fragile epidermis of young embryos may have been a factor in the decreased frequency of infection. In any case, the point to be emphasized is that embryonic rat skin representative of both extremes of gestation, as well as of intermediate stages, proved to be susceptible to infection with the Shope papilloma virus.

The animals bearing brain transplants rarely gave evidence of increased intracranial pressure, despite the presence of large tumors sometimes involving half a cerebral hemisphere. Occasionally, animals were found dead or were comatose when killed, but in such cases the transplants were not unusual in size, and it seems probable that the location of the tumor rather than increased pressure was the factor concerned. The presence of tumor was often evidenced by gross distortion of the brain, recognizable before section (Fig. 1). In other instances, removal of the cortex revealed masses of tumor bulging into the ventricle and easily dissectable from neighboring nervous tissue. Small tumors were most readily detected by palpation, their firm consistency contrasting sharply with the soft surrounding brain. In rare cases, the tumor was attached to the dura and the underlying brain compressed and indented by the growth.

The tumors were usually roughly rounded in contour but sometimes resembled casts of the ventricles, and hair was occasionally observed growing in long tufts from one or the other pole. On section the rounded tumors consisted of a central core of keratin surrounded by thick, fleshy tissue and resembled the dermal extensions of papillomas growing in intact adult rabbit skin. The more irregularly shaped growths contained cystic regions of similar appearance, together with areas grossly indistinguishable from transplants of normal uninfected skin. Histologically, the rounded tumors were made up entirely of papillomatous epidermoid epithelium and keratin, while the irregular growths contained interspersed areas of papillomatous and normal-appearing tissue (Figs. 2–6).

The possibility that conditions peculiar to the brain may have been factors in the susceptibility of embryonic rat skin grown in this site was tested by the subcutaneous transfer of infected embryonic skin. At the end of a month the transplants were equal in size to those grown in the brain and on section showed typical papillomatous lesions. The extent of papillomatous change was generally less than in brain transplants, and the lesion tended to be limited to portions of otherwise normal-appearing skin rather than widespread throughout the tissue (Figs. 7–10).

Serial transplantation of papillomatous tissue derived from brain and subcutaneous transplants was successfully effected in both sites, and there is no reason to believe that the tissue could not be maintained indefinitely by continued passage.

In view of the demonstrated susceptibility of embryonic rat skin to the papilloma virus, continued attempts were made to infect adult animals. The intact skin of newborn animals as well as of young and old adults was scarified and treated with cottontail virus, but no changes suggestive of the Shope papilloma were found on gross or histological study. As a further control, fragments of newborn and adult skin were treated with virus fluid and transplanted to the brains of 53 rats. The animals were killed at the end of a month, and, although growth occurred in the majority of cases, none of the transplants showed papillary changes (Fig. 11).

Guinea pig embryonic skin.—Twenty-three groups of embryos measuring from 1 to 9 cm. in

FIG. 1.—Brain of rat bearing transplant of embryonic rat skin treated with the Shope papilloma virus. The animal was killed 30 days after transfer, and the cortex of the right cerebral hemisphere has been removed to show the underlying transplant.

FIG. 2.—Rat brain bearing transplant of embryonic rat skin treated with the Shope virus. The animal was killed 1 month after transfer. The transplant has grown to occupy the greater part of a cerebral hemisphere. ×13.

FIG. 3.—Higher power view of transplant shown in Figure 2. ×60.

FIG. 4.—Transplant of embryonic rat skin treated with virus in brain of adult rat killed 25 days after transfer. ×40.

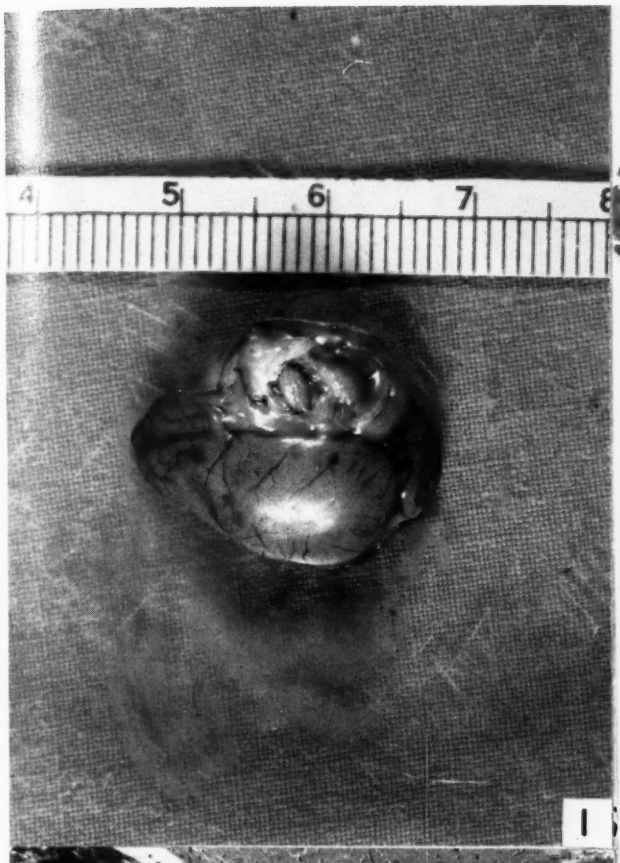
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FIG. 5.—Higher power view of transplant shown in Figure 4. ×140.

FIG. 6.—Control transplant of normal untreated embryonic rat skin in rat brain 30 days after transfer. Note keratin-filled cyst lined by epidermoid epithelium and dermis containing hair follicles and sebaceous glands. Compare with Figure 5 shown at same magnification. ×140.

FIG. 7.—Control transplant of normal untreated embryonic rat skin in subcutaneous space of adult rat killed 32 days after transfer. ×50.

FIG. 8.—Transplant of embryonic rat skin treated with Shope virus in subcutaneous space of rat killed 32 days after transfer. Compare with Figure 7. ×50.

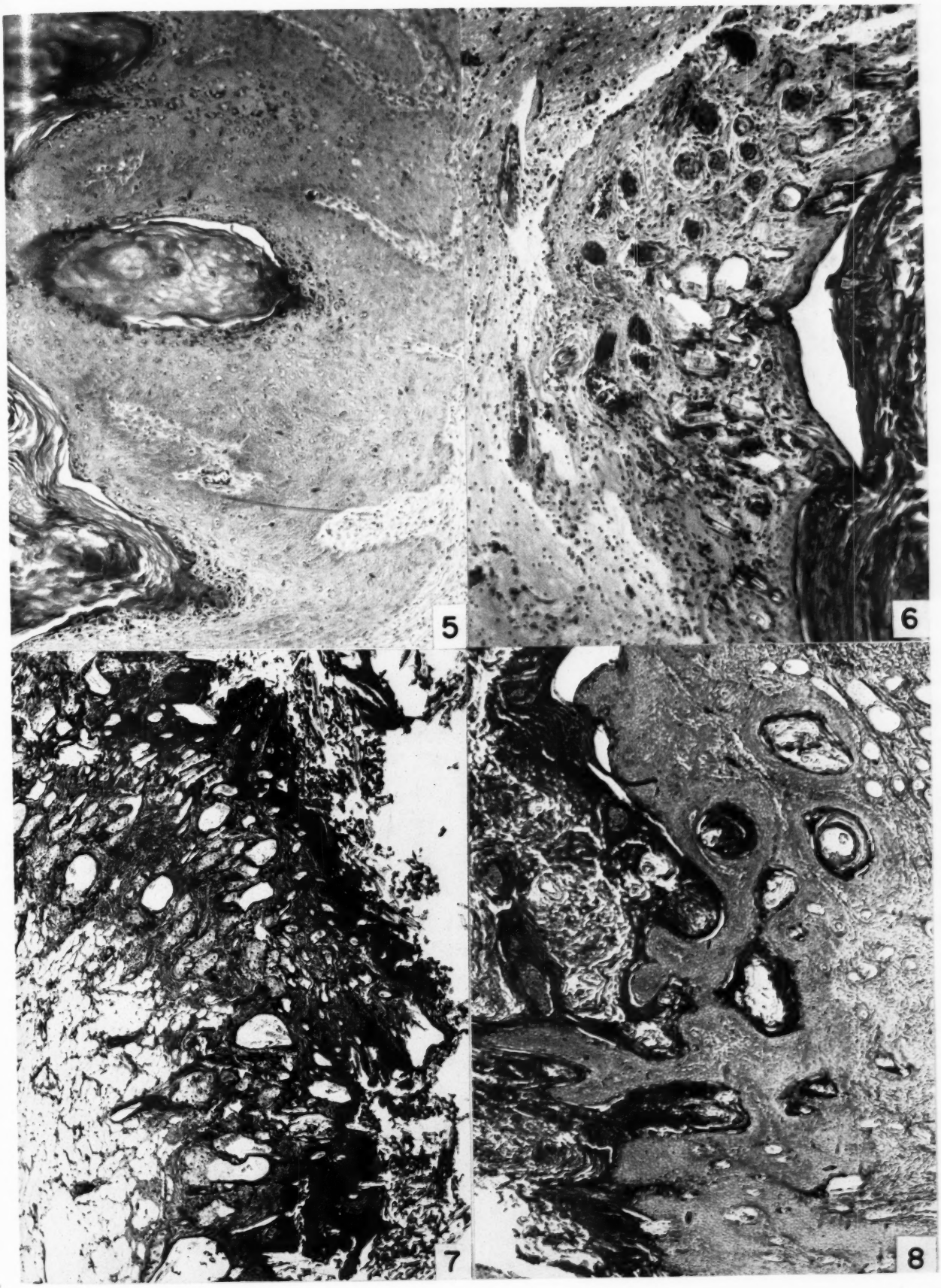
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FIG. 9.—Higher power view of transplant shown in Figure 8. ×140.

FIG. 10.—Transplant of embryonic rat skin treated with Shope virus in subcutaneous space of adult rat killed 28 days after transfer. ×110.

FIG. 11.—Transplant of adult rat skin treated with Shope virus in brain of adult rat killed 31 days after transfer. Keratin-filled cysts lined by flattened epidermoid epithelium with no evidence of papillomatous changes. ×150.

FIG. 12.—Transplant of embryonic guinea pig skin treated with Shope virus in brain of adult guinea pig killed 31 days after transfer. Note true papilloma without changes characteristic of the Shope lesion. ×75.

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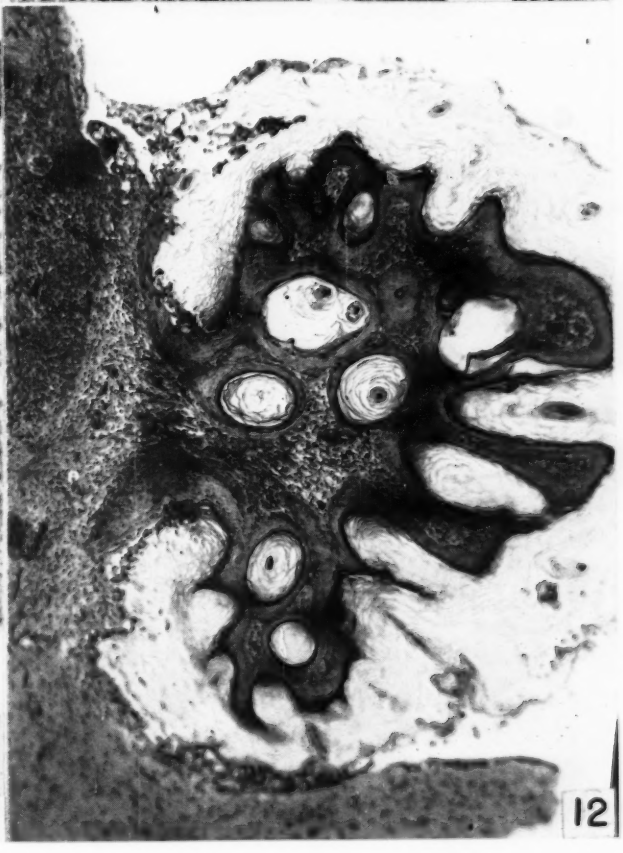

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length were used in these experiments, and transplants were made to the brains of 158 adult pigs. The animals were killed at the end of a month, and with few exceptions all bore growths. However, on histological study, the great majority consisted solely of normal skin and did not differ from control uninfected transplants. In three instances true papillomas were found. These were different in appearance from the lesions induced in rabbits by the Shope virus and consisted of a branched connective tissue stalk lined by epithelium without the intense hyperkeratotic changes characteristic of the Shope papilloma (Fig. 12). Their status in relation to the Shope virus has not been determined, but it is suggestive that in the scores of brain transplants of normal embryonic guinea pig skin examined such structures have never been observed.

In transplants derived from very young guinea pig embryos, scattered areas of hyperkeratosis bearing some resemblance to the Shope papilloma were occasionally noted. However, the lesions have not been found on serial transfer of such transplants, nor do the lesions show evidence of progression in animals held for 2 or 3 months.

Mouse and hamster embryonic skin.—More than 200 transplants of infected mouse skin derived from embryos measuring 0.5–2.5 cm. in length have been examined, and in no instance have suggestive changes been found. Similarly, 60 samples of skin derived from hamster embryos have been studied after treatment with the virus and transfer to the brains of adult hamsters without the finding of lesions resembling the Shope papilloma.

DISCUSSION

On a basis of the technics used in this study, it would appear that the resistance to the Shope papilloma virus characteristic of the adult guinea pig, hamster, and mouse is shared by their embryonic tissues, whereas the refractoriness shown by the adult rat is manifestly a developmental acquisition and not an attribute of the rat embryo. The transition from susceptibility to resistance in the latter species is abrupt and coincides with birth; prior to this event the skin is as susceptible as that of the rabbit, while after birth it is completely resistant.

The fact that embryonic rat skin undergoes growth and maturation to adult skin after transfer, yet reacts to the virus with the production of typical papillomas, suggests that the differentiation of embryonic and adult skin with respect to susceptibility and resistance relates to the infectability of the cell rather than to its ability to react to the infecting agent. In experiments of the present type the rat cell is infected when the embryonic cell is treated with virus, but at the time the morphological response occurs, the transplant has matured and is adult rather than embryonic in nature. Accordingly, it seems probable that the failure to induce papillomas in adult rat skin is due to a failure to infect the cells rather than to an inability of the cells to react to infection with the production of the specific lesion. The point is of interest inasmuch as it concerns the nature of the cellular change occurring at birth and indicates a direction of experimental approach. The question is open to investigation, and pertinent studies are in progress.

A basis accounting for the susceptibility of embryonic rat skin in contrast to the resistance of the embryonic skin of other species is not apparent. The fact that papillomas occur in embryonic rabbit skin treated with virus and transplanted to the brains of all the species involved (2) indicates that the factors differentiating the response of homologous embryonic skin reside in the tissue itself rather than in the constitution of the host species. Although no corresponding variation in histological structure or biological behavior is known, investigations in this direction will be continued.

In any case, the ability to induce the Shope papilloma in a species other than its natural host, the rabbit, offers a material for the study of a number of problems associated with the growth.

SUMMARY

Although the adult rat is resistant to the Shope papilloma virus, embryonic rat skin is susceptible. Fragments of embryonic rat skin infected with the virus and transplanted to the brains of adult animals react with typical papilloma formation. On the other hand, the refractoriness of the adult guinea pig, hamster, and mouse is shared by their embryonic tissues.

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The Effect of Antibiotics upon the Growth of Sarcoma 180 *in Vivo**

H. Christine Reilly, C. Chester Stock, Sonja M. Buckley, and Donald A. Clark

(Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, New York, N.Y.)

A review of the literature which is to be presented in a forthcoming paper (7) indicates that certain microörganisms have the ability to produce agents which interfere with the development of neoplastic cells. The diligent search among microörganisms for growth-inhibiting agents and the isolation of an ever increasing number of new antibiotics offer an excellent source of microbial products which possess specific biological properties. Although, as has already been stated, there is no reason to expect such materials to show a differential destructive effect between normal and cancerous cells, the possibility that some antibiotics may possess this property cannot be ignored. For this reason an attempt has been made to test many of the isolated antibiotics for their ability to retard the growth of Sarcoma 180 *in vivo*. Stock (9) has reported preliminary results on a small group of antibiotics; the data presented here comprise an extension of this study.

METHODS

Antibiotic preparations in various stages of purity were provided by various cooperating laboratories. Water-soluble agents were dissolved in sterile 0.85 per cent saline; water-insoluble ones were either suspended with grinding in sterile 0.5 per cent carboxymethyl cellulose in saline or dissolved in propylene glycol.

Preliminary toxicity information was either supplied by the source or was determined in our own laboratories.

The *in vivo* test procedure has been designed for the purpose of screening large numbers of materials for their ability to retard the growth of the mouse tumor, Crocker Sarcoma 180. It has been described in some detail elsewhere (11). Briefly, it consisted of the following: Small, uniformly cut pieces of a 7-day-old tumor were implanted subcutaneously in the axillary region of noninbred Swiss white mice weighing 18–22 gm.; each animal received one implant. The animals were divided into groups of five each. One group from each donor tumor was maintained as a control set and received injections of the vehicle used for solution or suspension of the agents being tested. Intraperitoneal administration of the preparations was begun 24 hours after implantation of the tumor. Materials in aqueous media were given in doses of 0.5 cc. twice daily for a total of thirteen injections; those in propylene glycol were administered in 0.05-cc. amounts once daily for 7 days. Results were not considered significant unless the animals were treated for at least 5 days.

On the 8th day after tumor implantation, the diameters of the tumors in all surviving animals were measured in two dimensions by means of calipers. The measurements for each group were averaged, and the sizes of the tumors in the antibiotic-treated mice were compared to those in control groups. The effect upon tumor development was graded according to the following arbitrary system:

$\pm$ = slight retardation; average diameter of treated tumors was $\frac{1}{4}$ to $\frac{3}{4}$ that of controls. Any slight effects reported in Table 1 were of a low order; the average diameters of the treated tumors were never less than $\frac{1}{2}$ those of control tumors.

$-$ = no effect; average diameter of treated tumors was $\frac{3}{4}$ or greater than that of controls.

All mice were weighed before start of treatment and again at the end of the experiment. Deaths resulting from toxicity are recorded.

Although in the routine screening procedure only five mice were employed for each test, in some instances when a preparation appeared to be of some special interest and was available in sufficient quantity, it was tested in a larger number of animals.

Whenever possible, if a negative effect was obtained with one concentration of material, the agent was retested as often as necessary at increasingly higher dose levels until a definite toxic effect upon the animals occurred. Occasionally it was possible to run only one test on an agent because the supply of material was limited.

RESULTS

The effects of 33 different antibiotics upon the growth of Sarcoma 180 in mice are recorded in Table 1. None of these agents, under the conditions employed, displayed such marked effects upon the development of the tumor as have been observed with other substances, such as the basic protein preparation from *Aspergillus fumigatus* (8), the folic acid analogs (10), 6-mercaptopurine (4), or 2,4,6-triethylenimino-*s*-triazine (3). Only one antibiotic, actidione, caused a measurable, although slight, retardation of tumor growth at a dose level which did not elicit toxic manifestations in the host. Although four other agents—namely, actinomycin, illudin M, illudin S, and terramycin, did exhibit slight retarding effects upon the tumor,

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TABLE 1

EFFECT OF ANTIBIOTICS UPON THE GROWTH OF SARCOMA 180 IN MICE

Antibiotic	Source*	Dose (mg/kg/day)	Vehicle†	S 180 inhibition‡	AWC§ (gm.)	Deaths#	Toxic dose‖ (mg/kg/day)
Actidione	BY, AE	50	2	±	0/0	0/15	100
Actinomycin	D	0.05	1	±	−3.0/+0.5	4/15	0.075
Aerosporin (Polymyxin B)	BB, BE	20	2	—	−1.5/−1.0	0/10	40
Antimycin A	DT	1	3	—	−1.0/0.0	2/10	
Aspergillic acid	AM	128	3	—	−1.0/0.0	0/5	150
Aureomycin	AG	250–300	2	—	−1.5/−1.5	1/10	500
Bacitracin	AL	450	2	—	−3.0/+1.0	1/10	
Borrelidin (Na salt)	AJ	2	2	—	−1.5/−1.0	24/50	5
Candicidin	EN	50	4	—	0.0/+1.5	0/5	100
Chloromycetin	C	1,000	3	—	0/0	2/10	1,500
Circulin HCl	BY	50	2	—	−0.5/−0.5	1/5	64
Citrinin	D	50	3	—	0.0/+2.5	2/10	60
Cordycepin	AW–5	165	3	—	−1.5/+1.5	3/10	
Dihydrostreptomycin SO_4	BB	1,000**	2	—	−1.5/−2.5	0/5	
Fumagillin	BY	35	3	—	−3.0/0.0	1/10	50
Fungicidin, crude	EN	100	2	—	−1.5/−2.0	1/5	
Gliotoxin	Z	2	3	—	−2.0/+1.5	1/10	4
Gramicidin	S	100	3	—	−2.0/+2.5	1/5	
Illudin M	AC	0.8	2	±	−3.0/−1.0	0/10	
Illudin S	AC	0.6	2	±	−4.0/−1.0	4/10	
Neomycin (180 units/mg)	BB	350	2	—	−1.0/−1.0	0/5	
Netropsin HCl	BB	4	3	—	+3.0/+1.0	1/10	8
Netropsin SO_4	BB	35	3	—	−2.5/+2.5	1/5	
Phagopedin sigma Na salt	AC–1	100	2	—	−2.0/−1.5	0/5	
Polypeptin	BQ	2	5	—	−1.0/−1.0	0/10	
Rimocidin	BB	30	3	—	−1.0/0.0	3/10	
Streptomycin SO_4	PD	500	2	—	−1.5/−1.0	1/10	1,000
Streptothricin HCl	D	35	2	—	−3.0/−1.0	0/5	50
Subtilin	D, BX	250	3	—	0/0	0/10	300
Terramycin base	BB	350	2	±	−1.5/−1.0	5/20	
Terramycin HCl	BB	250	2	±	−4.0/−1.0	2/15	350
Tyrocidine HCl	S	20	3	—	−1.5/−1.0	0/5	40
Tyrothricin	S	10	3	—	−2.0/+0.5	1/5	
Viomycin	BB	750	2	—	−2.0/−1.0	0/5	
Viscosin	EN	50	6	—	−0.5/−0.5	0/5	100

* AC Dr. Wm. Robbins, New York Botanical Gardens.
AC–1 Dr. I. N. Asheshov, New York Botanical Gardens.
AE National Research Council, Chemical-Biological Coordination Center.
AG Dr. J. H. Williams, Lederle Laboratories.
AJ Hoffmann-La Roche, Inc.
AL Ben Venue Laboratories.
AM E. R. Squibb & Sons.
AW–5 Dr. R. K. Barclay, Sloan-Kettering Institute.
BB Chas. Pfizer & Company, Inc.
BE Wellcome Research Laboratories.
BQ Dr. S. F. Howell, U.S. Public Health Service (Present address: Kirksville College of Osteopathy and Surgery).
BX Bristol Laboratories.
BY Upjohn Laboratories.
C Parke-Davis & Company.
D Merck & Company, Inc.
DT Dr. Curt Leben, University of Wisconsin
EN Dr. S. A. Waksman, Rutgers University.
PD Purchased.
S S. B. Penick & Company.
Z Wm. S. Merrell Company.

† 1 concentrated solution (1 mg/ml) in ethanol diluted in 0.85 per cent NaCl solution.
2 dissolved in 0.85 per cent NaCl solution.
3 suspended by grinding in 0.5 per cent carboxymethyl cellulose in saline.
4 dissolved in propylene glycol.
5 dissolved in 0.1 N HCl (1 mg/ml) and diluted with 0.85 per cent saline.
6 dissolved in 0.1 M phosphate buffer at pH 7.8.

‡ ± indicates slight inhibition; and — no effect. Explained in text under methods.

§ $\frac{\text{Average weight change of surviving treated mice}}{\text{Average weight change of control mice}}$.

\# $\frac{\text{Number of deaths}}{\text{Number of treated mice}}$ No deaths occurred in control groups during observation period.

‖ Dose resulting in death of 60 per cent or more of animals during treatment.

** Amount equivalent to 1,000 mg. of streptomycin.

these were achieved only at dose levels that were toxic for the mice, as was indicated either by death of some of the animals or by rather severe losses in body weight during treatment. Nontoxic levels of these substances were ineffective as antitumor agents.

That those slight effects on tumor development which were accompanied by toxicity were more or less specific in nature and not merely the result of generalized debilitation of the host is indicated by the fact that several agents—e.g., bacitracin, cordycepin, fumagillin, and gliotoxin, exerted no observable effect on the tumor at concentrations which were definitely toxic to the animals.

In addition to the testing of known antibiotics, some 1,256 preparations, which included crude microbial culture filtrates as well as partially purified substances, have been screened for tumor-inhibiting properties. Although all these materials were reputed by their contributors to be inhibitory for either bacteria, fungi, or viruses, only 92 produced any effect upon the growth of Sarcoma 180. Five of these, all crude filtrates, were sufficiently active to be worthy of further consideration.

DISCUSSION

As was to be expected in view of our knowledge of the highly specific nature of antibiotic action, there would appear to be no parallelism between the antimicrobial activity of such agents and their ability to interfere with tumor development. Since three of the agents—actidione, actinomycin, and illudin M—which did have a slight retarding effect on the tumor exert antifungal effects *in vitro*, a hasty conclusion might be drawn that there is a definite correlation between antifungal and antitumor activity. Further investigation, however, reveals that other antifungal agents, including antimycin A, candicidin, fungicidin, gliotoxin, and streptothricin, were ineffective in retarding Sarcoma 180.

The negative results obtained in the Sarcoma 180 screening program with most of the antibiotics reported here do not exclude the possibility that some of these agents may exert inhibitory effects upon other tumor types. Indeed, reports of the selective nature of the antitumor action of some compounds (6, 9, 10) emphasize the need for caution in drawing conclusions concerning the therapeutic potentialities of any substance from data obtained with only one type of tumor.

The slight effects obtained with some of the antibiotics inspire hope that a continued search for tumor-inhibiting agents among microbial products might well prove fruitful.

The data obtained with actidione indicate that this agent might be worthy of further investigation. In spite of the fact that a twofold increase in the dose which caused an effect on the growth of the tumor resulted in the death of about 80 per cent of the treated mice, it is felt that an intermediate dose level might show a more pronounced tumor retardation without causing severe adverse effects on the host. Such studies have been initiated. In addition, Bateman and Klopp (2) have reported preliminary studies on the use of combined aminopterin and actidione therapy in leukemic mice and in three clinical cases of neoplastic disease.

Asheshov *et al.* (1) have presented evidence indicating that fumagillin and phagopedin sigma are identical. It will be noted in Table 1 that the sodium salt of phagopedin sigma appeared to be much less toxic for mice than fumagillin. If these two antibiotics are the same, these results may indicate a real difference in biological activity between the two preparations, or they may be a reflection of variations in purity. Also, although the phagopedin sigma sample was tested very soon after its preparation, the possibility of its destruction during the experiment must be considered in view of the report of Eble and Hanson (5) that the potassium salt of fumagillin loses its antiphage activity upon standing.

SUMMARY

Thirty-three known antibiotics were tested for their ability to retard the growth of the Crocker Sarcoma 180 in mice. None is outstandingly effective. Five—namely, actidione, actinomycin, illudin M, illudin S, and terramycin—have slight retarding effects which, with the latter four agents, have been achieved only at doses toxic for the host.

Of more than 1,200 crude antibiotic preparations tested, only five caused sufficient retardation of tumor development to be considered for further work.

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Announcements

Medical Research Fellowships

The Division of Medical Sciences of the National Research Council is accepting applications for postdoctoral research fellowships for 1954–1955. These awards are designed to offer research experience for promising individuals who look forward to investigative careers, and not to provide practical experience in the clinical field. Ordinarily Fellowships are not granted to persons over 35 years of age. The following programs are announced:

Fellowships in Cancer Research are awarded by the American Cancer Society on recommendation of the Committee on Growth of the Division of Medical Sciences. Awards are available for study in all branches of the biological, chemical, and physical sciences and of clinical investigation applicable to the study of growth, typical or malignant. Citizens of the United States are eligible.

British-American Exchange Fellowships in Cancer Research also are awarded by the American Cancer Society upon recommendation by the Committee on Growth. They are offered to citizens of the United States for advanced study in Great Britain in specialized fields pertaining to the problem of cancer. Similar fellowships are awarded by the British Empire Cancer Campaign to young British scientists for research in the United States.

Fellowships in the Medical Sciences supported by The Rockefeller Foundation and by The Lilly Research Laboratories are administered by the Medical Fellowship Board of the Division. Fellows are expected to devote themselves to research in the basic medical sciences. The Rockefeller Fellowships are open to citizens of the United States and Canada; the Lilly Fellowships only to citizens of the United States.

Fellowships in Tuberculosis are also administered by the Medical Fellowship Board under a grant from the National Tuberculosis Association. These awards are designed to promote the development of investigators in fields related to tuberculosis. They are open to citizens of the United States who are graduates of American schools.

Fellowships in Radiological Research are administered by the Division's Committee on Radiology for the James Picker Foundation. The Foundation has expressed particular interest in the support of candidates who propose to carry on research oriented toward the diagnostic aspects of radiology. Appointments are not limited to citizens of the United States.

Applications for 1954–1955 under any of these programs must be postmarked on or before *December 10, 1953*. Fellowships are awarded in the late winter or early spring. Complete details and application blanks may be obtained from the *Fellowship Office, National Research Council, 2101 Constitution Avenue, N. W., Washington 25, D.C.*